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Award Number: DAMD17-96-2-6017

TITLE: A Novel Gene Gun-Mediated IL-12 Gene Therapy for Breast Cancer

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REPORT DATE: October 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Final (15 Sep 96 - 14 Sep 00)

4. TITLE AND SUBTITLE
A Novel Gene Gun-Mediated IL-12 Gene Therapy for Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-2-6017

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The results of this study show that particle-mediated IL-12 gene therapy was effective against mammary tumors in mouse models. IL-12 gene therapy of the immunogenic TS/A adenocarcinoma resulted in regression of the established primary tumors and induction of immunological memory. This IL-12 gene therapy protocol for the poorly immunogenic 4T1 adenocarcinoma did not significantly affect the growth of the primary tumor, but could reduce spontaneous metastasis into the lungs. The anti-metastatic effect of IL-12 was not mediated by T cells, but involved NK cells and interferon gamma. In combination with IL-12 gene therapy, IL-18 and ICE genes were found to be more effective in treatment of established TS/A mammary tumor than IL-12 alone. These results suggest that particle-mediated IL-12 gene therapy, alone or in combination with other immunological approaches, may be effective for treatment of breast cancer.

In a separate, but strategically relevant approach for cancer gene immunotherapy, a strong T cell-mediated antitumor effect was demonstrated in mice vaccinated with a gene encoding the tumor-associated antigen gp100 in combination with a GM-CSF gene. These results have served as the pre-clinical data for a first clinical trial using cutaneous particle-mediated gene transfer, initiated at the University of Wisconsin.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	37
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Dec. 5, 2000

PI - Signature Date

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A. INTRODUCTION

Evaluated by many clinical investigators, cytokine therapy has been actively considered as one of the most promising approaches for treatment of advanced forms of cancers, including breast cancer (1), because it can be directed at eradication of both the primary tumor and its metastases via activation of an antitumor immunity. Among various cytokines, interleukin 12 (IL-12) in particular exerted dramatic antitumor effects in several different experimental tumor models (2,3). Unfortunately, recombinant IL-12 protein in therapeutic doses can be toxic to mice and humans (4,5). Directed by the Army Breast Cancer Research Program, we have focused our laboratory effort on the potentially effective, and yet nontoxic, IL-12 gene therapy approaches for breast cancer.

The goal of this project was to develop an immunological approach for breast cancer gene therapy that can result in regression of both primary tumors and residual metastatic foci, and can also induce sufficient immunological memory to prevent tumor recurrence and progression. Based upon our previous studies using particle-mediated gene transfer (PMGT), this strategy was expected to exploit the gene therapy potential for treatment of breast cancer with minimal or none of the toxic side effects that have been previously encountered in other studies employing IL-12 protein therapy (4,5).

Our laboratories previously reported (6,7) that PMGT-mediated *in vivo* delivery of IL-12 DNA elicited effective antitumor responses with no evident toxicity. This therapeutic effect was achieved via localized transgenic production of IL-12 protein, at a systemic level at least 1,000 times less than the effective, and toxic, dose of recombinant IL-12 protein delivered systemically (8). We therefore suggested that particle-mediated IL-12 gene therapy might be effective and non-toxic in murine mammary tumor models. In our experiments we used two syngeneic mouse breast cancer models, TS/A and 4T1 adenocarcinomas, of which the histology, tumorigenicity, and metastatic capacity have been previously established (9-13). The results of our study, summarized below, suggest that the particle-mediated *in vivo* IL-12 gene therapy approach may be developed as an effective and safe alternative to systemic IL-12 protein therapy for breast cancer.

B. BODY OF REPORT

B1. Experimental Methods

cDNA Expression Plasmid Construction. We used two different expression plasmids for murine IL-12. Durinf first two years of the study, we used plasmid pWRG3169, which contains the coding sequences for the p35 and p40 subunits of murine IL-12 arranged in the same orientation in tandem, with each driven by its own CMV promoter. This and a luciferase (Luc) cDNA expression vector have been described by us previously (6). Later, a better expressing IL-12 plasmid, pNGVL3-mIL12, was constructed in a backbone plasmid containing a CMV immediate/early enhancer promoter, intron A and a kanamycin selection gene. The p35 and p40 subunits, separated by an internal ribosomal entry site, were subcloned into the multiple cloning site of pNGVL-3 (National Gene Vector Laboratory, University of Michigan, Ann Arbor, MI). The backbone plasmid, pNGVL-3, was used as a control vector. To construct a plasmid expressing pro-IL-18 or ICE cDNA, the full length of murine pro-IL-18 or ICE cDNA was obtained from total RNA of C57BL/6 mouse spleen cells by reverse transcription-polymerase chain reaction (RT-PCR) using an RT-PCR kit (Takara, Tsukuba, Japan). The PCR product of pro-IL-18 was subcloned into pNGVL-3 (pNGVL-3-mIL-18) and that of ICE was directly subcloned into the expression vector

pCR3.1 (Invitrogen, Carlsbad, CA, USA) by TA cloning (pCR3.1-ICE). For B16 cell transfection, the construct pNASS/CMV-hugp100 was used. It was generated by inserting the hugp100 DNA fragment, excised from the pWRG1644 vector, into the pNASS/CMV-neo vector.

Mice and Cell lines. BALB/c mice and C57Bl/6 mice were obtained from Harlan-Sprague-Dawley (Madison, WI), or from Taconic (Germantown, NY) and were housed in an AAALAC-accredited facility under isothermal conditions and allowed access to food and water ad libitum. Female mice between 6-12 weeks of age were employed. The cell lines were used in the studies included: TS/A (murine mammary adenocarcinoma), 4T1(murine mammary adenocarcinoma), Lewis lung carcinoma (LLC), B16 (murine melanoma), RAW 264.7 (murine macrophage) and COS-7 cells. TS/A, a moderately immunogenic tumor was obtained from Dr. G. Forni (Milan, Italy). 4T1, a poorly immunogenic tumor, was obtained from Dr. F.R. Miller (Michigan Cancer Foundation, Detroit, MI). The B16 melanoma cell line, syngeneic in C57Bl/6 mice, was obtained from Dr. William Ershler (UW Institute of Aging, University of Wisconsin-Madison). The cell cultures were maintained in RPMI 1640 (Biowhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% minimal Eagle's medium nonessential amino acid, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Biowhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO2 at 37°C.

Murine Tumor Models and In Vivo Treatment Protocol. Mice were shaved in the abdominal area and injected intradermally (i.d.) with $5x10^4$-$1x10^5$ tumor cells in 50 μl phosphate-buffered saline (PBS). We utilized a helium-pulse Dermal PowderJect-XR-1 (formerly Accell®) delivery device (PowderJect Vaccines, Inc., Madison, WI) as previously described (6). Briefly, plasmid DNA was precipitated onto gold particles 2 μm in size (Degussa, South Plainfield, NJ) at a loading rate of 2.5 μg DNA/mg of gold and coated onto the inner surface of Tefzel tubing. Each half-inch segment of tubing conferred the delivery of 0.5 mg gold and 1.25 μg total plasmid DNA per transfection. When two or three plasmids were used in combination, each plasmid was precipitated onto separate gold particles, which were then mixed together. In some experiments, hugp100 DNA was diluted 10-fold or 100-fold before precipitating onto gold particles. For DNA vaccination or treatment, mouse skin was shaved and transfected *in vivo* with the cDNA expression vectors with a 400 pounds per square inch (psi) helium gas pulse. For TS/A tumor treatment, mouse skin overlying and surrounding the tumor was transfected in with cytokine cDNA expression vectors and control plasmid (pNGVL-3) on days 6, 8, 10 and 12 after tumor implantation. There were six groups of treatments, which included: (I) mIL-12 cDNA alone; (II) murine pro-IL-18 cDNA alone; (III) murine pro-IL-18 and ICE cDNA (pro-IL-18 / ICE); (IV) mIL-12 and pro-IL-18 cDNA (IL-12 / pro-IL-18); (V) mIL-12, pro-IL-18 and ICE cDNA (IL-12 / pro-IL-18 / ICE); and (VI) control vector (pNGVL-3). Each treatment consisted of four transfections. Tumor growth was monitored two to three times per week by caliper measurement of two perpendicular tumor diameters. Mice were sacrificed when the diameter of the tumor reached 10 mm or greater. Mice that had been tumor free for more than 50 days after tumor implantation were challenged with an intradermal injection of 1×10^5 of the parental tumor cells (TS/A) or CT26 tumor cells (murine colon adenocarcinoma), which is also syngeneic to BALB/c mice.

***In Vitro* Gene Transfer and IL-18 Bioassay.** For *in vitro* transfection, 2.5×10^6 of COS-7 or TS/A cells were suspended in 30 μl RPMI-1640 and spread into the target size in a 35 mm dish. Transfections with cytokine cDNA were performed using the XR-1 gene transfection device with 250 psi helium pulse. Cells were then recounted using the trypan blue dye exclusion assay and 1×10^6 viable cells were placed in 2.0 ml of culture medium. Culture supernatants were harvested at

24 hr post-transfection for use in the IL-18 bioassay. The relative bioactivity of IL-18 was determined by the ability of cell supernatants to augment IFN-γ production *in vitro* (14). Briefly, mouse splenocytes (2 x 10^6) were cocultured with Con A (1.25 μg/ml) in 24-well plates for 48 hr. Supernatants obtained from transfected cells were added to cell suspensions of Con A-primed splenocytes (1 x 10^6) in 96-well plates for 24 hr. The supernatants were collected and assayed by ELISA to detect IFN- γ production.

In *Vivo* Depletion of $CD4^+$ and $CD8^+$ T cells and Neutralization of IFN- γ

The relative contribution of T cell subsets and IFN- γ was evaluated by *in vivo* antibody inhibition. Anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) were administered intraperitoneally at 0.3 mg per injection per mouse on days 5, 9 and 13 after tumor implantation (6). Anti-IFN-γ mAb (clone R4-6A2) was injected intraperitoneally at 0.5 mg per mouse on days 5, 7, 9, 11 and 13. As a control antibody, rat IgG (Sigma) was injected at 0.5 mg per mouse with the same schedule as for the anti-IFN- γ mAb.

Cytotoxic Assay. Spleen cells (8 x 10^6), derived from BALB/c mice that rejected TS/A tumors following gene therapy and remained tumor-free for at least 50 days, or obtained from TS/A tumor bearing mice 2 weeks after tumor implantation, were co-cultured with gamma-irradiated TS/A cells (4 x 10^5) for 5 days *in vitro*. TS/A cells labeled with $Na_2(^{51}Cr)O_4$ (5 x 10^3 / well) were cultured in a total volume of 200μl with effector cells in 96 round-bottomed well plate. After 4 hours of incubation, the supernatant was harvested and counted in a gamma counter, and specific lysis was calculated (6).

PCR. C57/BL6 mice were vaccinated using the PMGT method as described below. One transfection contained a total of 625 ng of DNA of either vector alone, or vector encoding hugp100, or vector encoding hugp100 mixed at a 1:1 ratio with vector encoding mouse GM-SCF. Twenty four hours later, skin samples were taken from the transfected area, and total RNA was extracted using the guanidinium method .

The RNA was quantified spectrophotometrically and tested for DNA contamination by running a PCR using 40ng of RNA as template and the primers listed below. All RNA was tested for DNA contamination, and RNA that showed signs of DNA contamination was subjected to DNAse treatment according to standard protocols. Single stranded cDNA was generated from RNA with oligo-dT priming and AMV-RT according to the manufacturers instructions (Promega, Corp., Madison, WI). The resulting cDNA was amplified using primers obtained from Integrated DNA Technologies Inc., Coralville, IA. One set of primers was designed to specifically amplify human gp100 (upper 5'-TATTGAAAGTGCCGAGATCC-3'; lower GCTGTTCTCACCAATGGGACAAG-3'), the other primer pair amplifies both human and mouse gp100 (upper 5'-TATTGAAAGTGCCGAGATCC-3', lower 5'-TGCAAGGACCACAGCCATC-3'). The conditions for the PCR reaction were: initial denaturation step 4 min at 94°C; 35 cycles of denaturation (94°C for 45 sec), annealing (55°C for 45 sec) and extension (72°C for 1.5 min), and a final extension at 72°C for 7 min. The products were evaluated by 1.4% agarose gel electrophoresis in ethidium bromide and visualized by UV light.

Flow cytometry. The parental (B16-wild) and hugp100-transfected B16 cells (B16-gp100) were first permeablized in ORTHO PermaFix™ solution (Ortho Diagnostics Systems, Raritan, NJ) for 1 hr, followed by incubating with anti-gp100 mAb, HMB-45 (15), obtained from Coulter-Immunotech (Westbrook, Me), for 40 min at 4°C. As negative control, MOPC IgG (Sigma, St. Louis, MO) was used. After washing, the cells were stained with FITC-conjugated goat-anti-mouse IgG (Becton Dickinson and Co., San Jose, CA) for 40 min at 4°C. Stained cells were

analyzed using a FACScan cytofluorometer (Becton Dickinson and Co., San Jose, CA) and data collected for 10,000 events/sample.

B2. Results

The results of this study have been presented in 1997,1998 and 1999 Annual reports. During the course of this study, all three proposed technical objectives and most of the tasks of the approved Statement of Work have been accomplished. In addition, some new research approaches directed at cancer gene therapy have been initiated and yielded interesting findings. Our findings are summarized as follows.

I. Antitumor effects of IL-12 gene therapy against primary breast tumors.

I.1. Immunogenicity of TS/A and 4T1 tumors.

Since human breast cancer, like most of the other types of cancer, is considered to be poorly immunogenic (16), we decided to employ for our study the poorly immunogenic murine mammary tumors. TS/A adenocarcinoma was described as non-immunogenic in some studies (9) and as poorly immunogenic in later studies by the same authors (13). We could not find a published reference on the immunogenicity of the 4T1 tumor, although it was personally communicated as non-immunogenic by Dr. F. Miller (Michigan Cancer Foundation, Detroit, Michigan). Therefore, we performed preliminary experiments to test the immunogenicity of these two adenocarcinomas. Balb/c mice were immunized twice at a 3-week interval by intradermal injection (i.d.) of 10^6 gamma-irradiated (5,000 rad) TS/A or 4T1 adenocarcinoma cells. One month after the last immunization, mice were challenged i.d. with 10^5 viable tumor cells, and tumor growth was followed. Figure 1 shows that whereas TS/A tumors did not grow in the mice pre-immunized with irradiated TS/A cells, 4T1 tumors grew in pre-immunized mice at the same rate as in control mice. Our data indicated, therefore, that TS/A is an immunogenic tumor, and 4T1 is non-immunogenic. However, since it is known that immunogenicity of a tumor may depend on the dosage and design of immunization or tumor challenge, we decided to perform a more detailed analysis of the immunogenicity of TS/A cells. Indeed, the results of two additional experiments show that when 10^5 4T1 tumor cells were used for a challenge, the protective effect of vaccination was not detected. However, the immune response could be detected when 10^4 or less tumor cells were used for challenge (ref. In D.2.4). Therefore, the 4T1 tumor can be classified as "weakly immunogenic", rather than "non-immunogenic". We have also obtained additional evidence confirming that low level immune response can be generated against the 4T1 tumor. Specifically, when mice immunized against TS/A tumors were challenged i.d. with 4T1 tumor several weeks later, a significantly reduced tumor growth was observed as compared with naïve mice challenged with the same dose of 4T1 cells. These results suggest that TS/A tumor and 4T1 tumor express shared tumor antigens, and that the immune response to 4T1 tumor can be induced if the immunogen is strong enough.

I.2. Effects of IL-12 gene therapy on the growth of primary TS/A and 4T1 tumors.

We then evaluated the antitumor effect of particle-mediated IL-12 gene therapy on established, intradermal TS/A and 4T1 tumors. The mice were treated according to a standard protocol that we previously established (6), starting on day 7 after tumor cell implantation. IL-12 gene therapy resulted in a substantial antitumor effect against the TS/A tumor (Fig. 2A), in that 50% of treated mice rejected established tumors (~4 mm average diameter at the start of treatment). This experiment was repeated twice more, and similar results were obtained. Also, similar to what we

reported in a previous study with other immunogenic tumors (6), mice that rejected TS/A tumors remained tumor-free for 2 months resisted a second challenge with the same tumor cells and were able to generate an augmented CTL response *in vitro* (ref. In D.2.4). In contrast to the TS/A tumors, the growth of the 4T1 primary tumors (Fig. 2B) was not affected by IL-12 gene therapy, which is consistent with the poorly immunogenic nature of this tumor.

II. Anti-metastatic effect of IL-12 gene therapy against 4T1 adenocarcinoma.

II. 1. Localized IL-12 gene delivery resulted in a systemic anti-metastatic effect against 4T1 tumor.

The 4T1 tumor line has been characterized as highly metastatic to the lungs and lymph nodes (11). During our experiments with therapy of intradermal primary tumors, as described above, we noticed that, on day 31 after tumor cell implantation, the Luc cDNA-treated (control) mice looked moribund, whereas the IL-12 cDNA-treated mice did not have visible signs of distress. When the mice were sacrificed and their lungs inspected, we noticed a striking difference in the size of the lungs and in the spread of metastatic nodules. The anti-metastatic effect of IL-12 gene therapy was further analyzed by weighing the lungs. Table 1 shows that the average lung weight in IL-12-treated mice was significantly lower than that of the Luc-treated mice or the untreated tumor-bearing mice. The observed effect against spontaneous metastases was further confirmed by two additional criteria: (a) the number of metastatic nodules in the lungs and (b) the survival time of mice following primary tumor excision.

To compare the number of tumor nodules in the lungs, mouse skin was transfected with either IL-12 cDNA or Luc cDNA on days 7,10,14,17 and 20 after 4T1 tumor cell implantation. Mice were sacrificed on day 21 and immediately injected intratracheally with India Ink followed by lung fixation in Feketr's solution as described (20). Mice treated with the Luc cDNA (n=8) were scored to contain 24.5 ± 3.8 visible tumor nodules in their lungs, whereas mice treated with the IL-12 cDNA (n=5) had 11.8 ± 1.3 tumor nodules ($p<0.01$).

For animal survival studies, we first performed an experiment to determine the time course of spontaneous metastasis of 4T1 tumor to the lungs. The primary tumors were surgically removed on day 7, 11 or 17 post tumor cell implantation (n=8 in each group), and survival time of the mice was followed. When the tumor was removed on day 7, 62.5% of mice survived for at least 120 days, whereas all mice with tumors removed on day 11 or 17 died by day 41 after tumor cell implantation. These results show that 4T1 tumors start to metastasize to the lungs during the first week of tumor growth, and that by day 11 virtually all mice have metastases that are destined to be lethal. Therefore, in the following experiments we excised the primary tumor on day 11 post tumor cell implantation. The results of two independent experiments on the time course of mouse survival are presented in Fig. 3. Mice were untreated or transfected in the skin overlying an intradermal tumor with the IL-12 cDNA or Luc cDNA expression vector on day 3 (Exp.1) or day 4 (Exp.2), and then on days 7 and 10 after 4T1 tumor cell implantation, followed by tumor excision. The combined data from these two experiments show that 5 of 28 mice (17.9%) treated with IL-12 cDNA survived the spontaneous metastases and were considered "cured", whereas only 1 of 28 (3.6%) in each of the control groups survived until day 120 (the final observation day of that experiment). This difference between the IL-12 group and the pooled control groups, 5/28 versus 2/56 surviving, was statistically significant by the Fisher Exact Test ($P = 0.038$). Among the mice that died during the course of the experiments, the IL-12-treated mice lived significantly longer than the Luc-treated or untreated mice (48.48 ± 2.43, 40.92 ± 1.52 and 37.14 ± 0.72 days, respectively; $p<0.025$). Mice that survived metastasis were, however, not able to resist a challenge

with 10^5 4T1 tumor cells (data not shown). Thus protective immunological memory was not induced.

In a separate experiment, we asked if the localized IL-12 gene therapy can result in the systemic antitumor effect against a distant solid tumor. 4T1 tumor-bearing mice were implanted intradermally with a secondary 4T1 tumor on the back, one day after the beginning of treatment of the primary, abdominal tumor with IL-12 cDNA. The growth of both primary and secondary tumor was followed. The localized IL-12 gene delivery resulted in a systemic antitumor effect, reducing growth of a secondary intradermal tumor (data not shown). These findings are in agreement with our data obtained from studies on systemic anti-metastatic effect of IL-12 gene therapy.

II.2. T cells are not required for the anti-metastatic effect of IL-12 gene therapy against 4T1 tumor.

We have previously reported (6) that the regression of immunogenic tumors induced by the current particle-mediated IL-12 gene therapy protocol is CD8+ T cell-dependent. However, the apparent inability of 4T1 tumor to induce a specific immune memory response as described above argues against the observed anti-metastatic effect of IL-12 gene therapy being due to a classical T-cell response. To directly assess the role of T cells, we injected 4T1 cells i.d. into athymic nude mice in a parallel experiment with the normal euthymic Balb/c mice, and treated the mice with our standard IL-12 gene therapy protocol followed by evaluation of lung metastases. Fig. 4A shows that IL-12 gene therapy resulted in a similar anti-metastatic effect in nude mice as can be obtained in euthymic mice, suggesting that T cells were likely not involved in the anti-metastatic effect observed in the 4T1 tumor model. This conclusion is supported by a T-cell depletion experiment in which 4T1 tumor-bearing mice were treated with either: (a) Luc DNA, (b) IL-12 DNA plus control immunoglobulin, or (c) IL-12 DNA plus antibodies to murine CD4+ and CD8+ T cells. The results in Figure 4B demonstrate that mice depleted of T cells were still able to suppress metastases in the lungs following IL-12 gene therapy, conforming the results previously obtained with nude mice. Together, these results strongly suggest that T cells do not mediate the IL-12-induced anti-metastatic effect against the 4T1 tumor.

II.3 Role of NK cells and IFNγ in anti-metastatic effect of IL-12 gene therapy against 4T1 tumors.

Since T cells do not play a major role in the observed systemic anti-metastatic effect of IL-12 gene therapy, and because IL-12 protein did not exhibit any inhibitory effect on the growth of 4T1 tumor *in vitro* (data not shown) or *in vivo* (Fig. 2), our working hypothesis was that transgenic IL-12 may have acted by stimulating or augmenting some local or regional nonspecific host defense mechanisms that could have actively suppressed 4T1 tumor metastasis. It has been shown previously that following subcutaneous implantation, 4T1 tumor first metastasizes into the regional lymph nodes and then into the lungs (11). We hypothesized, therefore, that IL-12 gene therapy may induce an immune or inflammatory reactivity in tumor-draining lymph nodes (TDLN) which in turn could inhibit tumor growth, invasion, or spreading.

To test this possibility, we first evaluated IFNγ production in TDLN, because it has been previously shown that IL-12 can readily stimulate IFNγ production (3,17), and IFNγ can be directly cytotoxic to tumor cells (18). Mice were injected i.d. in the middle of the abdominal area with 10^5 4T1 tumor cells, and skin-transfected with IL-12 cDNA or Luc cDNA 7 and 10 days later. On day 11, the draining (axillary) lymph nodes were removed, and the derived cells ($2x10^6$) were

incubated *in vitro* without any stimulation at 37°C for 48 hr. The cell-free supernatants (conditioned media) were assayed for IFNγ by ELISA. Fig. 5B shows that IL-12 gene therapy resulted in a substantially enhanced production of IFNγ in TDLN as compared with mice treated with Luc cDNA, or untreated tumor-bearing mice. In addition, it was found that recombinant mIFN γ at doses equal or above 1 ng/ml could significantly inhibit proliferation of 4T1 cells *in vitro* (data not shown).

We also evaluate NK cell activity in TDLN cells following IL-12 gene therapy, because it has been reported previously that IL-12 augments both the cytotoxicity of NK cells (19) and the IFNγ production by NK cells (20). Mice were injected i.d. in the middle of the abdominal area with 10^5 4T1 tumor cells, and skin-transfected with IL-12 cDNA or Luc cDNA 7 and 10 days later. On day 11, axillary lymph nodes were removed, and the derived cells were directly assayed for NK cytotoxicity against YAC-1 cells. The results in Figure 5A show that NK activity in TDLN of IL-12 gene-treated mice was higher than in control mice.

Since increased activity of NK cells and augmented level of IFNγ were detected in TDLN from mice that received IL-12 gene therapy, we next tested whether NK cells or IFNγ were involved in the IL-12-induced anti-metastic effect against 4T1 tumors. To do this, mice received injections of either anti asialo GM1 Ab, or anti-IFNγ mAb, respectively, starting one day before the treatment with IL-12, and continued throughout the treatment. The results in Fig.6Ashow that the anti-metastatic effect of IL-12 gene therapy was reduced in NK cell-depleted mice. Similarly, neutralization of endogenous IFNγ in vivo resulted in partial suppression of the IL-12-induced anti-metastatic effect in the lungs (Fig.6B)

III. Combinational (IL-12, pro-IL-18, ICE) Gene Therapy Approach.

III.I. Induction of IFN- γ by gene transfer of IL-12, pro-IL-18 and/or ICE.

To augment the antitumor effect of IL-12 gene therapy, we next decided to test the combination of IL-12 DNA with other effective cytokine genes. A combination of IL-12 and interleukin 18 (IL-18) proteins has been previously demonstrated to confer a superior antitumor activity over either IL-12 or IL-18 treatment alone (21,22). However, the systemic administration of recombinant IL-12 and IL-18 proteins, though effective in tumor growth inhibition, resulted in death of all treated animals due to toxicity (22). In this study, we evaluated and characterized the combination of IL-12, pro-IL-18 and ICE cDNA expression vectors for cancer gene therapy using particle-mediated gene transfer. ICE is the enzyme required for converting a natural, biologically inactive form of IL-18 (pro-IL-18) into a functionally active IL-18 molecule.

We hypothesized that expression of endogenous ICE in tumor cells may result in cleavage of the transgenic pro-IL-18. To evaluate this hypothesis, we transfected COS-7 cells (ICE negative) and TS/A (ICE positive) cells with pro-IL-18 cDNA alone or in combination with an ICE cDNA expression vectors. The ability of these cells to stimulate IFN- γ production in Con A-primed murine splenocytes was utilized as a bioassay to measure the relative levels of functional IL-18 protein (14-16). Fig. 7 shows that IFN- γ level induced by transfection with pro-IL-18 and ICE was considerably higher than that by pro-IL-18 alone in both COS-7 and TS/A cells. As expected, the cotransfection of ICE and pro-IL-18 cDNA was superior to pro-IL-18 cDNA alone and resulted in enhanced bioactivity of IL-18 in the ICE negative cell line COS-7 (23). Both the low level of endogenous ICE in TS/A cells and subsequent low level of IFN- γ production, however, were significantly enhanced by ICE cDNA cotransfection. Thus, dual transfection of pro-IL-18 and ICE

cDNA results in secretion of more bioactive IL-18 protein than that of pro-IL-18 cDNA alone. The combination of IL-12, pro-IL-18 and ICE resulted in the highest level of IFN- γ release from Con A-primed splenocytes among all groups in COS-7 and TS/A cells (Fig.7). In contrast, the IFN- γ level induced by pro-IL-18 and IL-12 together was not statistically different from that of treatment with IL-12 alone. Taken together, in terms of IFN- γ induction, IL-12 was synergistic with the IL-18 produced from ICE-cleaved pro-IL-18, but not with pro-IL-18 alone. Similar results were obtained in transfection studies performed with LLC and B16 cells (data not shown).

To investigate whether IFN- γ levels could be augmented in vivo following the transfer of IL-12, pro-IL-18 and ICE cDNA, IFN-γ production in the gene-treated skin tissues and serum of test mice and in conditioned medium of cultured splenocytes were compared among various treatment groups. The IFN- γ level produced from the skin in the group of IL-12 / pro-IL-18 / ICE was the highest among all tested groups (Fig. 8A). When splenocytes freshly isolated from test mice were cultured for 24 hours in the absence of Con A, the level of IFN- γ secretion was greatest in the group that was treated with the set of IL-12, pro-IL-18 and ICE cDNA in combination (781 pg / 10^6 splenocytes / 24 hours, Fig. 8B). This result suggests that splenocytes can be significantly activated following in vivo gene delivery of IL-12, pro-IL-18 and ICE, and that particle-mediated skin transfection with IL-12, pro-IL-18 and ICE cDNA may be capable of stimulating immune cells systemically as well as locally.

III.2. Antitumor effect of PMGT of IL-12, pro-IL-18 and ICE cDNA.
The highest level of IFN- γ induction via the combination of IL-12, IL-18 and ICE treatment among all tested sets, *in vitro* and *in vivo*, led us to test whether this combination could also result in regression of established TS/A tumors. The combination gene therapy of IL-12, pro-IL-18 and ICE elicited the most marked suppression of tumor growth among all groups (Fig. 9). Even more important, a complete regression was observed on day 50 in 11 out of 22 tested mice (50.0%) in the IL-12 / pro-IL-18 / ICE combinatorial gene therapy group, and this rate was 2- to 3-fold higher than that obtained from treatment with IL-12 alone, or IL-12 / pro-IL-18 (Table 2). These results further confirm that the combination of IL-12 / pro-IL-18 / ICE in this gene therapy strategy can confer a much more efficacious antitumor activity than that from either the IL-12 gene alone or IL-12 / pro-IL-18 genes. This is the first evidence that a combinatorial direct treatment with IL-12 and IL-18 cDNA can result in complete eradication of established and relatively large tumors.

III.3. Involvement of CD8+ T cells and IFN- γ in Tumor Regression Following the Combinatorial Gene Therapy of IL-12, pro-IL-18 and ICE. To determine the mechanism of cytokine-mediated tumor regression, in vivo depletion experiments were performed by injecting TS/A tumor-bearing mice with anti-CD4+, anti-CD8+ or anti-IFN- γ mAb (Fig. 10). Mice treated with IL-12 / pro-IL-18 / ICE and both anti-CD4+ and CD8+ mAbs were found to develop tumors with kinetics similar to that of the control plasmid group (Fig. 10A). In vivo depletion of CD8+ T cells, but not of CD4+ T cells, completely abrogated the antitumor effect of combination therapy (Fig. 10B). These results suggest that tumor regression induced by this combinatorial therapy requires CD8+ T cells.

In vivo neutralization of IFN- γ proteins with anti-IFN- γ mAb also inhibited the antitumor activity against the TS/A tumor induced by IL-12 / pro-IL-18 / ICE cDNA, although this inhibitory effect was not complete (Fig. 10A).

III.4. CTL Activity Induced by PMGT of Cytokine cDNAs. To further evaluate whether the mice that showed complete regression of tumor following in vivo gene transfer were able to

develop antitumor immunity, splenocytes collected from mice with complete regression in each treatment group were assayed for CTL activity. Splenocytes from IL-12 alone, IL-12 / pro-IL-18, and IL-12 / pro-IL-18 / ICE gene-treated mice generated similar levels of CTL activity, which were 4- to 6-fold higher than those from control tumor-bearing mice ($p < 0.01$, Fig. 11).

III.5. Immunological Memory in Mice Following Gene Therapy of IL-12, pro-IL-18 and ICE. We evaluated whether the mice that showed complete regression of TS/A tumors following gene therapy of IL-12 / pro-IL-18 / ICE could develop tumor-specific immunity. Six Balb/c mice with complete regression of TS/A tumor following in vivo PMGT of IL-12 / pro-IL-18 / ICE cDNA were rechallenged on day 50 with 1×10^5 TS/A cells. All mice rejected TS/A tumors and were tumor-free for an additional one month. Then, these mice were re-challenged again with 1×10^5 of both TS/A cells and CT26 cells (syngeneic to BALB/c mice) on the right and the left side of abdomen, respectively. All mice rejected a second challenge with TS/A tumor cells but developed CT26 tumors (data not shown). Taken together with the data of the cytotoxic assay (Fig. 11), our results suggest that PMGT with combination of IL-12, pro-IL-18 and ICE in tumor-bearing mice can effectively result in the CTL activation and the development of tumor-specific immunological memory.

IV. Gp100+GM-CSF Gene Vaccination Approach

IV.1. Hugp100 expression *in vitro* and *in vivo* in B16 tumors and in the skin following particle-mediated hugp100 DNA transfection. In parallel with the experiments described above, we were investigating a separate approach for cancer gene immunotherapy, in which mice are vaccinated with a gene coding for tumor-associated antigen. In the case of weakly immunogenic tumors, such as the 4T1 adenocarcinoma, genetic vaccines may have a potential to induce T cell immune response. As a model tumor antigen, we have chosen the melanoma-associated antigen gp100. A weakly immunogenic murine B16 melanoma stably transfected with human gp100 cDNA was used as the test tumor model.

Before evaluating antitumor efficacy of gp100 DNA vaccination, it was necessary to determine whether cell transfection with the hugp100 cDNA expression plasmid results in effective production of transgenic hugp100 protein. B16 cells were transfected with hugp100 cDNA, selected in vitro for positive clones (B16-gp100), and then analyzed for hugp100 expression using flow cytometry. Wild type B16 cells (B16-wild) and B16 cells transfected with empty vector (B16-neo) were used as controls. Because gp100 is a transmembrane glycoprotein, cell membrane was permeablized prior to staining with hugp100-specific HMB-45 mAb. The results in Figure 12A show that B16-gp100 cells expressed more gp100 than control mouse B16 melanoma cells, indicating that the B16-gp100 clone expresses hugp100 . The fact that nontransfected mouse B16 cells were also stained with HMB-45 mAb strongly suggests that this mAb recognizes an epitope shared between human and mouse gp100, since these proteins have approximately 80% homology in amino acid sequence (24) . A non-melanoma murine cell line, 4T1 adonocarcinoma, was not stained with HMB-45 mAb (data not shown).

In a second approach, we looked at the expression of hugp100 in B16-gp100 cells by using RT-PCR analysis. Several previously published primer sets for the human gp100 sequence proved unsuitable in our system because of their cross-reaction with the mouse gp100, which has a 79.7% homology with the human sequence (24). We therefore designed a new set of primers that is able to amplify only the human gp100 from hugp100-transfected, mouse gp100-positive cells. RT-PCR tests using the human-specific primer set confirmed it's specificity, in that only the human gp100

transfected cell line, B16-gp100, resulted in an amplification product (Fig 12B, Lane 2) but not B16-wild (Fig. 12B, Lane 1).

In order to confirm the transcription of DNA that is delivered via PMGT into the skin of mice, C57/BL6 mice were treated with empty vector as a control, or with hugp100 DNA-coated gold beads, or with a mixture of hugp100 and GM-CSF- coated beads. The mice were given one transfection on each side of the abdomen, using 625 ng (hugp100) or 1250 ng (hugp100+GM-CSF) of total DNA per transfection. Twenty four hours later, skin samples were removed from the transfected area and total RNA was extracted. The RNA was tested for DNA contamination and then reverse transcribed and amplified by PCR using primers for the house keeping gene G3PDH and the hugp100. Lane 3 in Fig.12B shows that no hugp100 was detected in the empty vector-transfected skin, indicating that the specificity of the primers for the hugp100 sequence is high and no mouse derived products are interfering with the human-specific primers. A clear band in Lane 4 shows the positive amplification product of hugp100- transfected skin. Lane 5 confirms the expression of hugp100 when transfected along with GM-CSF, indicating that GM-CSF does not interfere with the expression of hugp100 in the skin. The integrity of all the cDNA samples was tested and confirmed by amplifiing the house keeping gene G3PDH (Panel b). Thus, these data show that hugp100 DNA vaccination results in transgene transcription in the skin tissues 24 hr post- transfection.

IV.2. Protection against B16-gp100 tumor following gp100 gene vaccination

The skin of mice was transfected at four abdominal sites with hugp100-encoding plasmid DNA, alone or in combination with mGM-CSF-encoding plasmid DNA. This resulted in a total delivery of 2.5 μg of each DNA. Seven days later, vaccinated or naive mice were challenged i.d. in the middle of the abdomen with $5x10^4$ of either B16-wild cells, or B16-gp100 cells, and tumor growth was followed. No significant tumor protection was observed against wild-type B16 tumor (Fig.13, left panel), or B16-neo tumor (data not shown) following either gp100 or gp100+GM-CSF gene vaccination. In contrast, gp100 gene vaccination resulted in a substantial protection against B16-gp100 tumors, with 40% of mice remaining tumor-free for at least two months. Importantly, co-delivery of GM-CSF cDNA with hugp100 cDNA resulted in a complete tumor protection in all 5 vaccinated mice (Fig. 13, right panel). This ability of GM-CSF gene co-delivery to enhance the effect of gp100 DNA vaccination was consistently reproducible in all twelve subsequent experiments, although the degree of protection varied.

IV.3. Dose effect of gp100 cDNA gene vaccination.

Because of the observed enhancing effect of GM-CSF on gp100 gene vaccination, we determined next whether the reduction of gp100 cDNA dose in a combined DNA vaccine would still result in tumor protection. The vaccination was designed as follows: the initial gp100 DNA dose (625 ng per transfection) was decreased 10-fold and 100-fold, while the GM-CSF DNA dose remained constant (625 ng per transfectioin). Control mice received a vaccination with the empty vector alone and in combination with GM-CSF DNA. Seven days post vaccination, mice were challenged with $5x10^4$ B16-gp100 cells, and tumor growth was followed. The results in Fig.14 show that, in combination with GM-CSF gene, a 10-fold reduced dose of gp100 plasmid DNA (62.5 ng) still induced an appreciable level of protection, which was less than that induced by 625 ng of gp100 DNA+ GM-CSF, but still much higher than gp100 DNA alone. Fig.14 also shows that the protection against B16-gp100 tumors, although substantial, was not strong enough to completely eradicate the tumor in all vaccinated mice, in that some mice eventually developed the tumors several weeks after the tumor challenge.

IV.4. Role of T cells in tumor protection in gp100 + GM-CSF DNA –vaccinated mice.

We investigated next whether T cells were responsible for the protection induced by gp100-GM-CSF gene vaccination. Mice were transfected in the skin with the GM-CSF-gp100 combined DNA vaccine on day 0, and injected i.p. with a mixture of anti-CD4 and anti-CD8 mAbs one day before and 5 days after B16-gp100 tumor challenge on day 7 post vaccination. Control mice received rat IgG. The results in Figure 15 show that *in vivo* depletion of T cells abrogated the protection induced by the DNA vaccine, indicating that this protection is T cell mediated. In agreement with this result, we also observed that the vaccinated mice that remained tumor-free after the tumor challenge for more than a month, were partially or completely immune to a secondary tumor challenge (data not shown).

IV.5. RT-PCR analysis of recurrent tumors in vaccinated mice.
Although gp100 gene vaccination, especially in combination with GM-CSF, resulted in tumor protection, tumors relapsed in some mice after several weeks post tumor challenge. It could be that B16-gp100 tumor cells injected into vaccinated mice have lost expression of hugp100 and, therefore, became resistant to hugp100-specific T cell immune response. To address this question, we analyzed the expression of hugp100 RNA in tumors that developed in vaccinated mice at different times after the first or second tumor challenge. The RT-PCR analysis results presented in Fig.16 show that tumors which developed in vaccinated mice shortly after the challenge, 4 weeks after the challenge, or even 4 weeks after the second challenge, continued to express hugp100 RNA.

IV.6. Therapy of established tumors with the hugp100-GM-CSF DNA vaccine.
The experiments described above showed that gp100 gene vaccination of naive mice, especially in combination with GM-CSF, can result in induction of a T cell-mediated immune response which is capable of protecting mice from a subsequent tumor challenge. To approximate the experimental conditions to clinical situation, we investigated the therapeutic effect of the GM-CSF-gp100 gene therapy in mice bearing established tumors. Mice were injected i.d. in the middle of the abdomen with B16-gp100 tumor cells. Seven days later, when the tumors reached about 4 mm in diameter, mice received a combined GM-CSF-gp100 treatment at two sides of abdomen. This treatment was repeated on day 10, 13 and 17 post tumor cell implantation. The results in Fig.17A show that the GM-CSF-gp100 gene combination induced suppression of tumor growth when compared to control mice ($P<0.05$ starting from day 17 of tumor growth).

The effect of gp100-GM-CSF gene therapy against established tumors was further confirmed by the extended survival of mice. The survival was calculated based on the days the mice were sacrificed when their tumor diameter reached 15 mm. Thus, untreated tumor-bearing mice and mice treated with the empty vector + GM-CSF cDNA survived for 26.11 ± 0.96 and 25.5 ± 1.42 days, respectively, whereas gp100 $\pm$ GM-CSF cDNA-treated mice survived for 37.5 ± 2.72 days ($P<0.005$). The experiment depicted in Fig.5 was repeated two times, with the treatment started on day 4 or on day 7 post tumor cell implantation, and similar results were obtained.

IV.7. Vaccination with hugp100+GM-CSF DNA induces antitumor effect against a wild type B16 tumor.
The primary purpose of these studies was to measure the influence of GM-CSF DNA administration on the immunity induced by hugp100 DNA vaccination, as measured by protection from challenge with B16-gp100 cells. As the hugp100 molecule is cross-reactive with the murine gp100 molecule, we also tested whether this hugp100 + GM-CSF DNA vaccination regimen induced any antitumor effect against the wild type murine B16 melanoma, which expresses the murine gp100 molecule. Although a single vaccination was not protective against the challenge with the wild type tumor cells (Figure 13, left panel), 3 vaccinations with gp100 + GM-CSF DNA

induced a statistically significant ($P<0.025$) inhibition of growth of wild type B16 tumor as compared with control mice (Figure 17B).

B3. Discussion

I. Antitumor and anti-metastatic effects of IL-12 gene therapy.

The leading cause of death of women with breast cancer is tumor metastases in visceral organs (1,16). Recent progress in tumor and cellular immunology has provided good evidence that human breast tumors express tumor associated antigens (TAA), such as HER-2/neu (25), p53 (26) and DF3/MUC-1 (27), which are capable of inducing cytotoxic T cell responses in patients (16). As a result, immunotherapeutic strategies for treatment of metastatic breast cancers have received increased attention in recent years. One such approach involves the use of recombinant cytokines with the purpose of boosting the existing antitumor immune response. Studies in animal models have shown that IL-12, when compared with other cytokines, has outstanding antitumor efficacy (2,3,7), as was expected based on its known immune stimulatory effects on Th1 cells, cytotoxic T-cells and natural killer (NK) cells (28,29). Based on these studies, clinical trials with IL-12 were initiated. Unfortunately, the first clinical trial protocol revealed substantial toxicity of recombinant IL-12 protein in humans, resulting in the death of two patients and severe side effects in 15 others (5). Although the lack of a pre-dosing schedule was suggested as the cause for this toxicity, recent experiments have also suggested a need to explore alternative IL-12 delivery mechanisms, to assure that the treatment is both safe and effective (30). Using an IL-12 gene therapy approach, we have shown that localized *in vivo* IL-12 gene transfer into skin tissue can result in eradication of established murine tumors and their metastases, leading to the generation of a strong tumor-specific immunological memory (6). More importantly, no signs of IL-12 toxicity were observed following this particle-mediated therapy protocol (8). Therefore, we suggested that the particle-mediated IL-12 gene therapy protocol may be effective in treatment of metastatic breast cancer. The results of this study show that, indeed, *in vivo* PMGT of IL-12 DNA can result in complete regression in 50% of cases of the immunogenic, metastatic murine TS/A adenocarcinoma growing intradermally. When a poorly immunogenic, metastatic 4T1 tumor was employed in similar experiments, the growth of intradermal tumors was not affected, but a significant reduction of lung metastases was observed in 4T1 tumor-bearing mice as a result of this IL-12 gene therapy protocol. We believe that extension of this study may provide an experimental rationale for proceeding to a clinical trial of particle-mediated IL-12 gene therapy for breast cancer.

The understanding of tumor immunogenicity is important for developing rational design of cancer immunotherapy in humans. In classical animal studies, a tumor is considered to be immunogenic if it induces, following either surgical excision or vaccination, protection against a secondary tumor challenge *in vivo* (31). However, tumors originally classified as "non-immunogenic" by this criterion, after being biologically or transgenically modified, were often able to induce an immune response even against the unmodified tumor cells (32-35). The murine tumors used in this study, TS/A and 4T1 mammary adenocarcinomas, were tested for immunogenicity during the first year of study; we found the TS/A tumor to be immunogenic, and 4T1 to be non-immunogenic based on the ability of tumor vaccine induce a protection against a single-dose tumor challenge. We suggested then that a tumor which is considered to be non-immunogenic based on immunization-challenge experiments, such as the 4T1 adenocarcinoma employed in our study, may be later characterized as poorly immunogenic if tumor cells, or the experiment conditions, could be modified to induce an immune response. Indeed, by reducing the dose of tumor challenge we were able to demonstrate a low level protection against 4T1 tumors in vaccinated mice (Figure 1). In addition, we found that TS/A tumor-vaccinated mice were able to suppress growth of 4T1 tumor

challenge as compared with naive mice (data not shown). It is evident, therefore, that under certain conditions it may be possible to induce immune response against 4T1 tumor.

Even though 4T1 tumor may induce a low level immune response, we show in this study that the anti-metastatic effect against the 4T1 tumor is not T cell-mediated (Figure 3). This suggests that IL-12 fails to induce a therapeutic level of T cell immunity against this poorly immunogenic tumor. These data are in contrast with the T cell-dependent antitumor effect of IL-12 gene therapy against immunogenic tumors (6), but are in agreement with the studies using some other cytokine gene therapy strategies, which showed that the antitumor responses in a number of cases may not involve T cells (33).

The mechanisms of the anti-metastatic effect of IL-12 gene therapy in the 4T1 tumor model are not clear at this time. It appears that T cell activities including T cell-mediated cytotoxicity are not involved, since an anti-metastatic effect of similar magnitude was also observed in T cell deficient (both nude and T cell-depleted) mice. Knowing that 4T1 cells metastasize from a subcutaneous deposit first to TDLN and then into lungs and other organs (11), we hypothesized that some local or regional processes, induced by transgenic IL-12, may be responsible for the reduction of tumor metastasis into the lungs. Indeed, we found an increased NK activity and IFNγ production in TDLN of IL-12-treated mice (Figure 4). This is in agreement with reports of others showing that NK cells and IFNγ are induced by IL-12 (3,17,19,20) and can confer direct cytotoxicity to tumor cells (18). These results suggest that the immune activation in TDLN induced by IL-12 gene therapy may restrict or inhibit tumor metastasis from the primary tumor site into the lungs.

Our results show that systemic effect of IL-12 gene therapy can be detected not only against metastases in the lungs, but also against secondary intradermal tumors (Figure 6). It would be interesting to know whether both of these secondary tumors are controlled by the same or different mechanisms. It is possible, for example, that although T cells are not crucial against lung metastases, they may play a role in suppression of intradermal secondary 4T1 tumors. We are planning to perform in vivo depletion experiments and immunohistological studies to address these questions.

Compared to IL-12 protein therapy, we are delivering much lower systemic levels (~1000-fold) in a localized manner at the tumor site. Another distinct feature of the gene therapy approach is that we are achieving a slow, continuous release of IL-12 over a period of several days. Thus it is not clear whether the observed anti-metastatic effect in the lungs is due solely to the presence of the transgenic IL-12 protein, or may also be due to the slow, continuous release achieved via the epidermal gene transfection. For example, it is possible that the slow release of localized, transgenic IL-12 protein can activate regional immunity in a different way than the bolus injection of rIL-12 protein which would rapidly diffuse under *in vivo* conditions. The mechanism of the observed phenomenon - an efficacious anti-metastatic activity in the absence of T cell immunity - hence warrants further investigation.

We suggest, based on this and previous studies (6-8), that particle-mediated *in vivo* IL-12 gene therapy may be further developed as an effective and safe alternative approach to systemic IL-12 protein therapy. The TS/A and 4T1 mammary tumor cell lines used in this study, and the different antitumor effect of IL-12 gene therapy on primary tumors versus visceral metastases, may offer a highly desirable experimental model for immunotherapy and gene therapy studies related to human breast cancer. Our specific strategy for future research is to increase the anti-tumor immune response by increasing tumor cell immunogenicity via *ex vivo* transfection with IFNγ, GM-CSF, or B7-1 genes. Indeed, in our preliminary experiments 4T1 tumor cells transiently transfected with

IFNγ DNA induced a slightly stronger tumor protection than a nonmodified 4T1 vaccine, or sham-transfected 4T1 vaccine (data not shown).

III. Combinational (IL-12, pro-IL-18, ICE) Gene Therapy Approach

In order to augment the antitumor effect of IL-12 gene therapy, we attempted to combine IL-12 gene therapy with other immunomodulatory genes. Combination of IL-12 cDNA with IL-10 or Lymphotactin cDNA proved to be only marginally effective or noneffective (data not shown). The triple combination of IL-12, pro-IL-18 and ICE cDNA was much more effective than each treatment alone in suppressing the TS/A tumor growth (Figure 8, Table 1). These results are in agreement with the study showing a synergistic antitumor effect of IL-12 and IL-18 (22). It is also noteworthy that we show that the triple gene set with IL-12, pro-IL-18 and ICE in combination can be successfully delivered *in vitro* and *in vivo* by PMGT with a gene transfection device. In addition, in terms of induction of IFNγ the finding that transfection of pro-IL-18 and ICE cDNA is superior to that of pro-IL-18 cDNA alone suggests that the two testing genes were concomitantly expressed in the same cell, because cleavage of pro-IL-18 by ICE requires the expression of both transgenes within the same cell (23,36). Thus, PMGT effectively results in the expression of at least two or perhaps three transgenes in the same cells under both *in vitro* and *in vivo* conditions. Technically, this technique enables multiple gene transfer and expression in the same cells by co-precipitation of multiple species of DNA molecules onto the same gold beads (37,38).

Previous reports demonstrated the efficacy of a combination of IL-18 and IL-12 protein therapy in a murine model. Although tumor regression was noted, the treatment related toxicity was very high (21). In contrast, no adverse effect was observed in treated mice in current experiments using the combination gene therapy protocol. This suggests that particle-mediated local delivery of IL-12 / pro-IL-18 / ICE genes may be clinically desirable and safe as a strategy for local cancer therapy.

Results from our present study also demonstrate that IL-12 gene therapy can induce substantially more potent antitumor effects on the established tumor than the IL-18 gene therapy. In TS/A tumor-bearing mice receiving PMGT of IL-12 cDNA, 17.4 % of mice showed a complete regression of the established tumors. In contrast, IL-18 gene therapy as administered here has failed to eradicate tumors completely and all IL-18 cDNA treated mice eventually died from progression of testing tumors (Table 1). Others have also reported that the antitumor effects of SCK cells (murine mammary adenocarcinoma) expressing IL-12 were more striking than those of SCK cells expressing IL-18 in tumor protection model (22). Importantly, we also showed in this study that the potent antitumor effects induced by IL-12 / pro-IL-18 / ICE cDNA were in fact reflected by the prolongation of survival time in treated animals. When mice whose tumor did not exceed 100 mm^2 in size were defined as survivors, in the groups of mice receiving gene therapy with IL-12 / pro-IL-18 / ICE, pro-IL-18 /IL-12, and IL-12 alone, 59.1%, 30.4% and 17.4% of mice were found to survive on day 50, respectively.

Our finding that the combinational gene therapy requires mainly CD8+ T cells is similar to the results of antibody depletion experiments for animals receiving IL-12 protein or gene therapy alone (2,6). Micallef et al. demonstrated that the effector cells responsible for antitumor effects of pretreatment with rIL-18 were NK cells in the initial phase, both CD4+ and CD8+ T cells in the second phase (between day 9 and 5), and CD4+ T cells for the long term immunological memory (41, 42). Our results indicate that the combination therapy of IL-12 and IL-18 can confer antitumor effects primarily via activation of CD8+ T cells.

Result of IFN- γ induction experiments suggests that IFN- γ is, in part, responsible for the

antitumor efficacy of the combinatorial gene therapy with IL-12 / pro-IL-18 / ICE. The lack of complete abrogation of antitumor efficacy in animals treated with anti-IFN- γ Ab may be explained by persistent local IFN-γ even in the mice treated with anti-IFN- γ Ab. Alternatively, it is likely that IFN- γ may be an important, but not necessarily essential intermediate in this antitumor cascade. This is supported by studies showing that the use of IFN- γ gene in similar tumor treatment models has not resulted in tumor regression (43), implying that IL-12 and this combinatorial gene therapy can confer immunologically mediated antitumor effects via mechanisms other than those induced by IFN- γ.

In summary, PMGT of expression vectors for IL-12, pro-IL-18 and ICE cDNA can confer a synergistic induction of IFN- γ *in vitro* and *in vivo*. Combination of these three genes can effectively result in complete regression of TS/A tumors, which effect is superior to either IL-12 or pro-IL-18 cDNA alone. This antitumor response can be completely blocked by CD8+ T cell depletion, and partially abrogated by antibodies to IFN- γ. In addition, this combinatorial gene therapy can induce tumor-specific immnological memory. To our knowledge, this is the first report on the successful use of a triple gene combination for cancer gene therapy using non-viral vectors. These findings suggest that combinatorial gene therapy of IL-12, IL-18 and ICE cDNA may provide a potential application for cancer gene immunotherapy, and the current particle-mediated gene delivery approach may provide a new methodology for effective and functional delivery of multiple, candidate therapeutic genes for experimental and potential clinical applications.

IV. Gp100+GM-CSF Gene Vaccination Approach

Genetic vaccination against cancer seems to have a great potential as a simple and yet effective way of inducing a protective immune response. We show in this study that particle-mediated vaccination of mice with hugp100 plasmid cDNA , especially in combination with mGM-CSF cDNA, resulted in effective protection against hugp100-expressing melanoma. Importantly, as low as 62.5 ng of hugp100 plasmid was sufficient to achieve a substantial level of tumor protection when combined with the mGM-CSF plasmid.

Immunostimulatory peptides of hugp100 have been recently synthetized and modified to become more immunogenic (24,41). These peptides were found to induce a protective immune response in cancer patients (42,43). Remarkably, an initial study has indicated that 42% of melanoma patients demonstrated objective responses as a result of gp100 peptide vaccination followed by a course of treatment with recombinant IL-2 (44). Thus, vaccination against the gp100 antigen may offer an effective approach for melanoma treatment. Another way to achieve an immune response against gp100 is via genetic immunization with gp100 cDNA. DNA immunization could potentially be more effective than peptide immunization, as has been recently indicated in a comparative experimental study using a model tumor antigen (45). Genetic vaccination with naked hugp100 plasmid DNA resulted in generation of CTL and protection against hugp100-expressing B16 tumors (46). Our results confirmed these findings and further demonstrated that in order to achieve comparable antitumor effect, the PMGT method required 20 times less hugp100 DNA (2.5 μg) than needed for intramuscular injection of naked DNA (46). These results may have important clinical implications.

Our data demonstrate, in addition, that the effective dose of gp100 DNA can be reduced 40 times by co-administration with the gene encoding for mGM-CSF. An adjuvant effect of GM-CSF DNA for experimental cancer vaccination has previously been reported (47). The mechanism of this adjuvant effect may be attributed to the ability of GM-CSF to induce and activate antigen-presenting cells, such as dendritic cells and macrophages, and thereby potentiate a specific immune

response elicited by tumor vaccines. At least in some tumor systems, GM-CSF was found to be the most potent adjuvant as compared with other cytokines genes (35). In agreement with those findings, our preliminary results indicate that GM-CSF is more potent in enhancing the protective effect of gp100 DNA vaccination than IL-4 or B7.1 DNA (data not shown).

A theoretical disadvantage of a vaccination approach based on using a specific TAA peptide or plasmid DNA encoding a TAA is the possibility of tumor escape from the immune control by developing "antigen loss" or antigen-negative tumor variants. Indeed, in a clinical study using gp100 peptide vaccination, it was observed that a patient initially responded to the vaccination by tumor shrinkage but later the tumor relapsed (48). This relapsed tumor has lost the expression of gp100 and became resistant to gp100-specific CTL. Our data on experimental gp100 gene vaccination also provide evidence that test tumors may escape from the T cell-mediated immune control. However, we found that the relapsing tumors, being insidious for several weeks in vaccinated mice, continued to express hugp100 RNA. Although this observation does not exclude the loss of gp100 at the protein level, it is highly unlikely that the tumor cells would develop a new mechanism to regulate a gene on a post-transcriptional level rather than by simply deleting it. It is possible, instead, that when the immune response to gp100 weakens below certain threshold, dormant tumor cells from a challenge inoculum may begin to grow. Future experiments are warranted to determine the mechanism of tumor escape in gp100-vaccinated mice.

C. KEY RESEARCH ACCOMPLISHMENTS.

- Particle-mediated IL-12 gene therapy of the immunogenic TS/A adenocarcinoma can result in complete regression of some of the established primary tumors and induction of immunological memory.

- This IL-12 gene therapy protocol for the non-immunogenic 4T1 adenocarcinoma does not significantly affect the growth of primary tumors, but can reduce their metastasis into the lungs.

- The current IL-12 gene therapy can significantly extend the test mouse survival time following the excision of a 4T1 primary tumor.

- The observed anti-metastatic effect of IL-12 gene therapy against the 4T1 tumor apparently did not involve T-cells, but appeared to involve NK cells and IFN-γ.

- Co-delivery of a combination of IL-12, pro-IL-18 and IL-1b converting enzyme (ICE) cDNAs resulted in a synergistic and significantly enhanced inhibition of the growth of TS/A tumors.

- The IL-12, pro-IL-18 and ICE combinational gene therapy can result in high level of IFN-γ induction in vitro and in vivo, and is apparently CD8+ cell dependent.

- Using a DNA vaccine approach, co-delivery of GM-CSF cDNA together with gp100 cDNA into skin reproducibly resulted in a much higher level of protection against challenge with a murine B16 melanoma stably transfected with human gp100 cDNA.

- Seven scientific papers including one book chapter have been generated from this study. Moreover, the results of this study have served as the basis of pre-clinical data support for a

first clinical trial using particle-mediated gene transfer initiated at the University of Wisconsin by our collaborators.

D. REPORTABLE OUTCOMES.

D.1. PRESENTATIONS AT THE MEETINGS

1. Rakhmilevich, A.L., K.Janssen and N-S. Yang. Gene gun-mediated IL-12 gene therapy for breast cancer in mice. Cancer Gene Therapy, 4:S52-S53, 1997. A poster presentation at 6th International Conference on Gene Therapy of Cancer, Nov 20-22, 1997, San Diego, CA.

2. Rakhmilevich, A.L., K.M.Wright, B.Javorsky, T. Roberts, M. R. Albertini, N-S. Yang, and P.M.Sondel. Pre-clinical In Vivo Gene Therapy Approaches Using Gene Gun. An oral presentation at 7th International Conference on Gene Therapy of Cancer, Nov 19-21, 1998, San Diego, CA.

3. N-S. Yang, A.L.Rakhmilevich, P.M.Sondel, D.Mahvi, G.Hogge, and G.MacEwen. Cytokine gene therapy for cancer and DNA cancer vaccines: A gene gun approach. An oral presentation at 1998 Keystone Symposium "Synthetic Non-Viral Gene Delivery Systems", January 19-25, 1998.

4. N-S. Yang, K. Oshikawa, D. Mahvi, F-S. Shi, M.Imboden, M.D.Macklin, M.R.Albertini, T.Roberts, K.M.Wright, P.M.Sondel, and A.L.Rakhmilevich. Gene gun-mediated DNA cancer vaccination with combinational cytokine gene therapy, and tumor-associated antigen and GM-CSF. 2nd Annual Meeting, American Society of Gene Therapy, June 9-13, 1999.

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D.2. PUBLICATIONS

1. Rakhmilevich A.L., Timmins J.G., Janssen K., Pohlmann E.L., Sheehy M.J. and Yang N-S. Gene gun-mediated IL-12 gene therapy induces antitumor effects in the absence of toxicity: a direct comparison with systemic IL-12 protein therapy. *J. Immunother.*, 22, 135-144 (1999)

2. Oshikawa K., Shi F., Rakhmilevich A.L., Sondel P.M., Mahvi D.M., and Yang N-S. Synergistic Inhibition of Tumor Growth in a Murine Mammary Adenocarcinoma Model by Combinational Gene Therapy Using Interleukin-12, pro-Interleukin-18 and IL-1b-Converting Enzyme cDNA. *Proc. Natl. Acad. Sci. USA*, 96:13351-13356 (1999).

3. Rakhmilevich A.L. and Yang N-S. In vivo particle-mediated gene transfer for cancer therapy. In *Methods in Molecular Medicine, Vol.35: Gene Therapy: Methods and Protocols*, editors W.Walther and U.Stein, Humana Press, Inc., Totowa, NJ, 331-344 (2000).

4. Rakhmilevich A.L., Janssen K., Hao Z., Sondel P.M. and Yang N-S. Interleukin 12 gene therapy of a weakly immunogenic mouse mammary carcinoma results in reduction of spontaneous lung metastases via a T cell-independent mechanism. *Cancer Gene Therapy*, 7:

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5. Rakhmilevich A.L., Imboden M., Hao Z., Macklin M.D., Roberts T., Wright K.M., Albertini M.R., Yang N-S and Sondel P.M. Effective particle-mediated vaccination against mouse melanoma by co-administration of plasmid DNA encoding gp100 and granulocyte-macrophage colony-stimulating factor (submitted).

6. Oshikawa, K., Shi, F., Rakhmilevich A.L., Sondel P.M., Mahvi D.M., and Yang N.S.Particle-Mediated IL-12 Gene Transfer into the Skin Distant from Tumor Site Elicits Anti-metastatic Effect Equivalent to Local Gene Transfer. *Human Gene Therapy* (in press).

7. Shi F., Heise C.P., Oshikawa K., Sondel P.M., Rakhmilevich A.L., Yang N.S., and Mahvi D.M. Intratumoral injection of IL-12 plasmid DNA, either naked or in complex with cationic lipid, results in similar tumor regression in a murine model. (submitted).

D.3. FUNDING APPLIED FOR

1. American Cancer Society Institutional Research Grant ADevelopment of a new strategy for genetic immunization against melanomas≅, $15,000 award, 1998-1999. PI-A.L.Rakhmilevich

2. MACC (Midwest Athletes Against Childhood Cancer) Fund Grant "Genetic Immunization Against Cancer Using Heat Shock Protein", $50,000 award, 2000-2001. PI-A.L.Rakhmilevich

3. UWCCC Pilot Grant "Modulation of gp100-specific immunity by anti-CD40 antibody", $20,000 award, 2000-2001. PI-A.L.Rakhmilevich

4. U.S. Army MRMC Idea Award Grant "Combination of Anti-VEGFR-2 Antibody and Tumor Vaccine for Treatment of Experimental Metastatic Breast Cancer", $300,000, pending. PI-A.L.Rakhmilevich

5. U.S. Army MRMC Clinical Bridge Award Grant "Treatment of Experimental Breast Cancer with Combination of IL-12 Gene Therapy and Anti-VEGFR-2 antibody", $300,000, pending. PI-A.L.Rakhmilevich

6. NIH RO1 grant: Experimental immunotherapy of melanoma via CD40 ligation. 1999. Not funded. PI-A.L.Rakhmilevich

7. U.S. Army MRMC Idea Award Grant "Combination of Anti-VEGFR-2 Antibody and IL-12 gene therapy for Treatment of Experimental Metastatic Breast Cancer", 1999. Not funded. PI-A.L.Rakhmilevich

8. Concern Foundation grant "Combination of Anti-VEGFR-2 Antibody and IL-12 gene therapy for Treatment of Experimental Metastatic Breast Cancer",1999. Not funded. PI-A.L.Rakhmilevich

E. CONCLUSIONS

- IL-12 gene therapy of the immunogenic TS/A adenocarcinoma results in regression of established primary tumors and induction of immunological memory.

- IL-12 gene therapy of the poorly immunogenic 4T1 adenocarcinoma does not significantly affect the growth of the primary tumor, but reduces spontaneous metastasis into the lungs.

- Anti-metastatic effect induced by IL-12 gene therapy against 4T1 mammary tumor is not T cell-mediated, but involves NK cells possibly via IFNγ-related mechanism.

- A brief course of IL-12 gene therapy can significantly extend mouse survival time following excision of a 4T1 primary tumor.

- A combinational gene therapy with IL-12, pro-IL-18 and ICE cDNA confers a synergistic effect against TS/A mammary tumor, resulting in extended survival of mice.

- A new approach for tumor gene immunotherapy by co-transfecting skin with the cDNA expression vectors encoding for GM-CSF and tumor-accociated antigen gp100, showed a promising antitumor effect.

- Tumor protection induced by gp100 + GM-CSF DNA vaccination depends on T cells.

- Skin delivery of gp100 + GM-CSF DNA can also result in suppression of growth of established mouse melanoma, followed by extended survival of treated mice.

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Table 1. Anti-metastatic effect of IL-12 gene therapy against a nonimmunogenic 4T1 tumor.

Exp	Treatment[a]	N (mice)	Lung Weight (mg)[b] Mean ± SEM	P
I	Luc	8	756 ± 63	<0.001
	IL-12	8	368 ± 36	
II	Luc	8	567 ± 66	<0.025
	IL-12	8	381 ± 30	<0.025
	None	8	544 ± 56	
III	Luc	8	593 ± 70	<0.01
	IL-12	7	363 ± 25	

[a] Balb/c mice were injected i.d. with 10^5 4T1 tumor cells. On days 7, 10, 13 and 16 post tumor cell implantation, skin overlying the tumor was transfected with 5 μg of IL-12 cDNA or Luc cDNA, or left untreated (Experiment II).

[b] On day 31 after tumor cell implantation, mice were sacrificed and lung weight was determined. Lung weight of age-matched naive Balb/c mice was 270 ± 25 mg (n=4).

Table 2. Complete regression of TS/A tumor following PMGT *.

Treatment	Mice with complete regression† / total	(%)
IL-12	4 / 23	17.4‡
pro-IL-18	0 / 15	0¶
pro-IL-18 / ICE	0 / 15	0¶
IL-12 / pro-IL-18	6 / 23	26.1§
IL-12/pro-IL-18/ ICE	11 / 22	50.0
Control (pNGVL-3)	0 / 23	0¶

*Treatment protocol was same as that of Figure 5 (in IL-12, IL-12 / pro-IL-18, IL-12 / pro-IL-18 / ICE and control, 7 to 8 additional mice were evaluated). †The number of mice in which complete regression of established TS/A tumor was observed. ‡, $P < 0.05$ versus IL-12 / pro-IL-18 / ICE; §, $P = 0.089$ versus IL-12 / pro-IL-18 / ICE; ¶, $P < 0.0001$ versus IL-12 / pro-IL-18 / ICE.

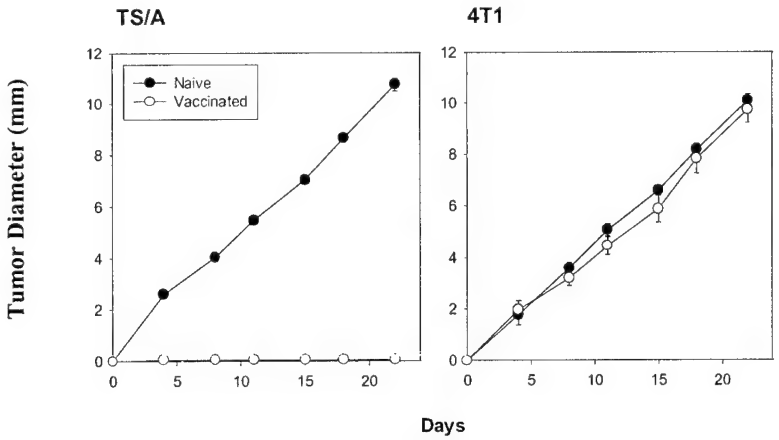


Figure 1. Comparative immunogenicity of TS/A and 4T1 adenocarcinomas.
Balb/c mice were injected i.d. in the right side of abdomen with 10^6 gamma-irradiated TS/A or 4T1 tumor cells twice in a 3 week interval. One month after the last immunization, vaccinated mice and naive mice were challenged i.d. in the left side of the abdomen with 10^5 viable, non-irradiated tumor cells, corresponding to the tumor used for vaccination, and tumor growth was serially measured. Mean tumor diameters ± SEM are shown for 7-8 mice per group.

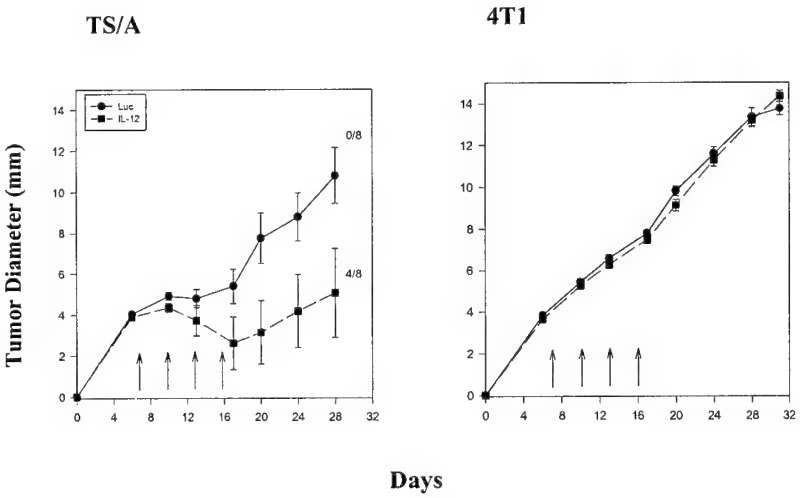
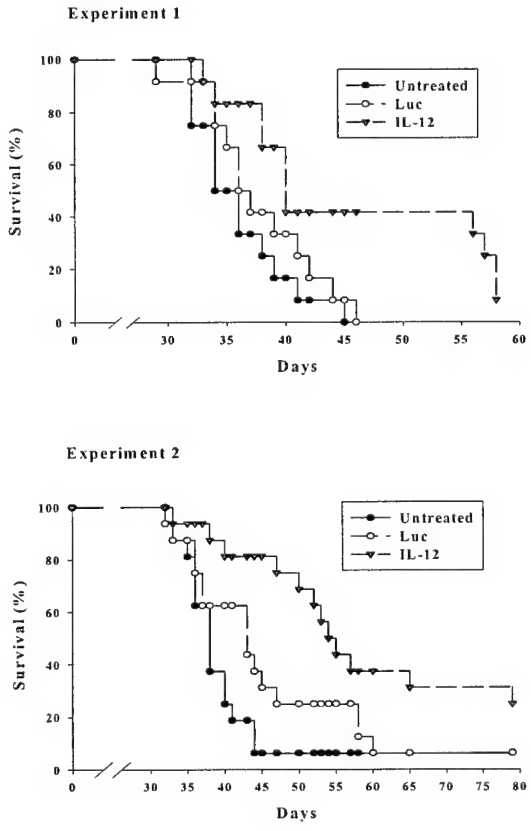


Figure 2. Antitumor effect of IL-12 gene therapy against intradermal TS/A and 4T1 tumors.
Mice were injected i.d. with 10^5 TS/A or 4T1 tumor cells. Gene therapy with IL-12 cDNA or Luc cDNA (control) was performed on days 7, 10, 13 and 16 post tumor cell implantation, as indicated by arrows. Numbers in the TS/A panel indicate mice with completely regressed tumors. Mean tumor diameters ± SEM for 8 mice per group.



Figire 3. Survival of mice from 4T1 tumor metastases after IL-12 gene therapy followed by excision of a primary tumor. Mice were injected i.d. with 10^5 4T1 tumor cells. Mice were untreated, or treated with IL-12 cDNA or Luc cDNA on days 3 (Exp.1) or 4 (Exp.2), 7 and 10 post tumor cell implantation, followed by the excision of the primary tumor on day 11. Survival of the mice was followed for 120 days. All mice that were alive after 58 days (Exp.1) or 80 days (Exp.2) remained tumor-free for the entire observation period. Data of two independent experiments are presented for 12 (Exp.1) and 16 (Exp.2) mice per group.

Figure 4. Anti-metastatic effect of IL-12 gene therapy against 4T1 tumor in nude mice (A) and in T cell-depleted mice (B). *A*. Euthymic Balb/c mice and athymic nude Balb/c mice were injected i.d. with 10^5 4T1 tumor cells. Gene therapy with IL-12 cDNA or Luc cDNA was performed on days 7, 10, 13 and 16 post tumor cell implantation. The lungs were removed on day 31, and their weight was determined. Mean lung weight ± SEM for 8 mice per group. *B*. Balb/c mice were injected i.d. with 10^5 4T1 tumor cells. Skin was transfected with IL-12 cDNA or Luc cDNA on days 7, 10, 13 and 17 post tumor cell implantation. A mixture containing 300 μg of each anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) was administered intraperitoneally on days 6,11 and 16 (experiment 1), or 6,10,15 and 20 (experiment 2) after tumor cell implantation. Flow cytometric analysis of peripheral blood cells of these mice, performed 4 days after the last mAb injection, confirmed depletion of 98% of $CD3^+$ cells. Control groups included mice that were treated with the IL-12 cDNA and received rat IgG (Sigma) at a dose of 600 μg/mouse per day following the same schedule as the anti-CD4/CD8 mAbs, or mice treated with the Luc cDNA instead of the IL-12 cDNA, or non tumor-bearing naive mice. The lungs were removed on day 31(experiment 1) or 34 (experiment 2), and their weight was determined. Mean lung weight ± SEM for two combined experiments.

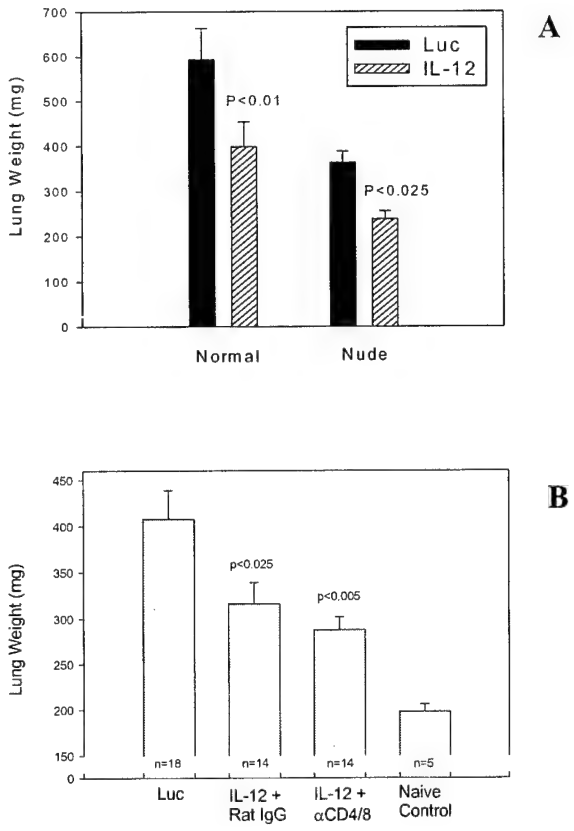
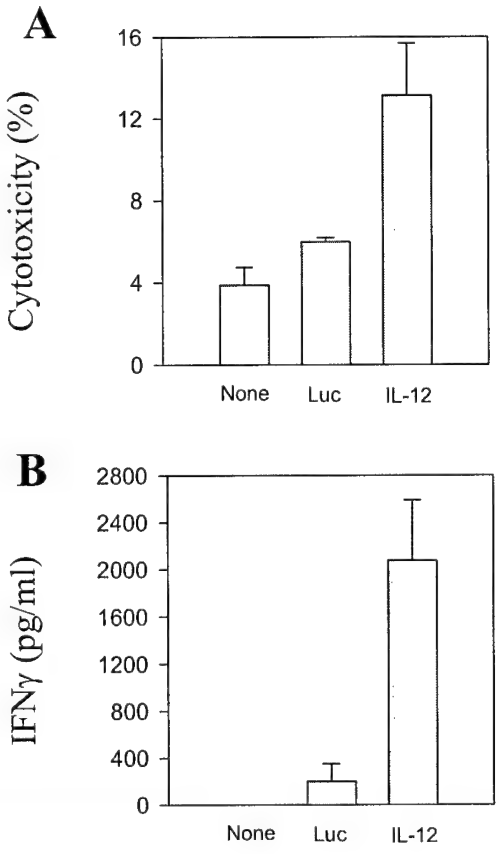


Figure 5. Immune activation of TDLN cells following skin transfection with IL-12 DNA. *A*. Balb/c mice were injected i.d. in the middle of the abdomen with 10^5 4T1 tumor cells. On days 7 and 10 post tumor cell implantation, skin overlying the tumor was transfected with 5 μg of IL-12 cDNA or Luc cDNA, or left untreated. On day 11 post tumor cell implantation, axillary lymph nodes were removed, pooled from 3 mice per group, and tested for cytotoxicity against NK-sensitive YAC-1 cells in a 4-hr ^{51}Cr assay. The results are expressed as cytotoxic index at the effector:target ratio 100:1. *B*. One million of the same lymphoid cells were placed in 1 ml of media for 48 hr. The activity of IFNγ in the supernatants was determined by ELISA.. Both graphs depict means ± SEM for two cell samples per group, each sample containing TDLN cells pooled from 3 mice.



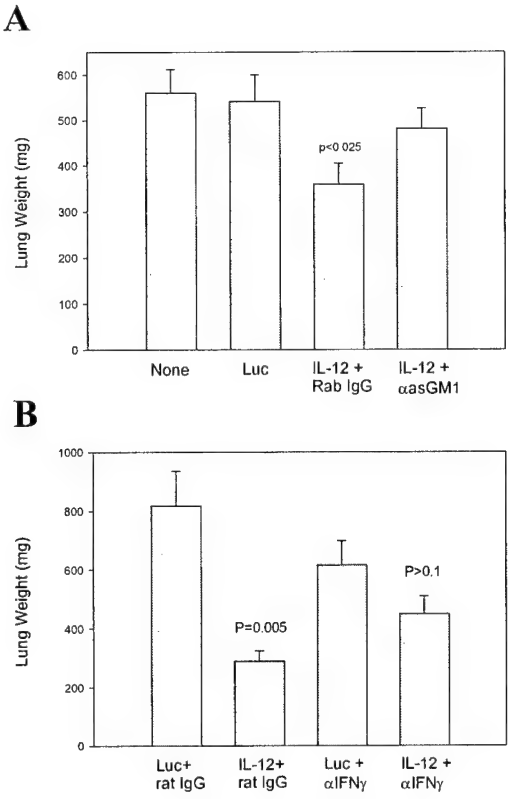


Figure 6. IL-12 gene therapy-induced anti-metastatic effect in mice treated in vivo with antibody against NK cells or IFNγ. *A.* Balb/c mice were injected i.d. in the middle of the abdomen with 10^5 4T1 tumor cells. On days 7, 10, 13 and 17 post tumor cell implantation, skin overlying the tumor was transfected with 5 μg of IL-12 cDNA or Luc cDNA, or left untreated. On day 6, 10 and 15 post tumor cell implantation, mice treated with IL-12 were injected via tail vein with 40 μl of anti asialo GM1 antibody, or with 1.5 mg rabbit IgG, in 200 μl PBS. Mice were sacrificed on day 33-36 post tumor cell implantation, the lungs were removed and their weight determined. Data shown are Mean ± SEM of 3 independent combined experiments, n=15-21 mice per group. P<0.025 represents a significant difference between the IL-12 + rab IgG – treated group and the nontreated group as well as the difference between the IL-12 + rab IgG – treated group and the Luc – treated group. The difference between the IL-12 + rab IgG – treated group and IL-12 + anti asialo GM1 antibody was not statistically significant. *B.* Mice were injected i.d.with 10^5 4T1 tumor cells. Because in this experiment tumors were developing at a slower rate than usually observed, initiation of gene therapy was delayed until day 11 and continued on days 14, 17 and 21 post tumor cell implantation. Mice treated with IL-12 cDNA or Luc cDNA were injected i.p. with 0.5 mg anti-IFNγ mAb (R4-6A2), or with 0.5 mg rat IgG, on days 10,12,14,16, and 18 post tumor cell implantation. The lungs were removed on day 39 post tumor cell implantation, and their weight determined. Results of a single experiment are shown as lung weights in mg (Mean ± SEM of 3-4 mice per group). P=0.005 represents a significant difference between the IL-12 + rat IgG –treated group and Luc + rat IgG –treated group. P>0.1 indicates no statistical difference between the IL-12 + αIFNγ mAb –treated group and Luc + αIFNγ mAb –treated group.

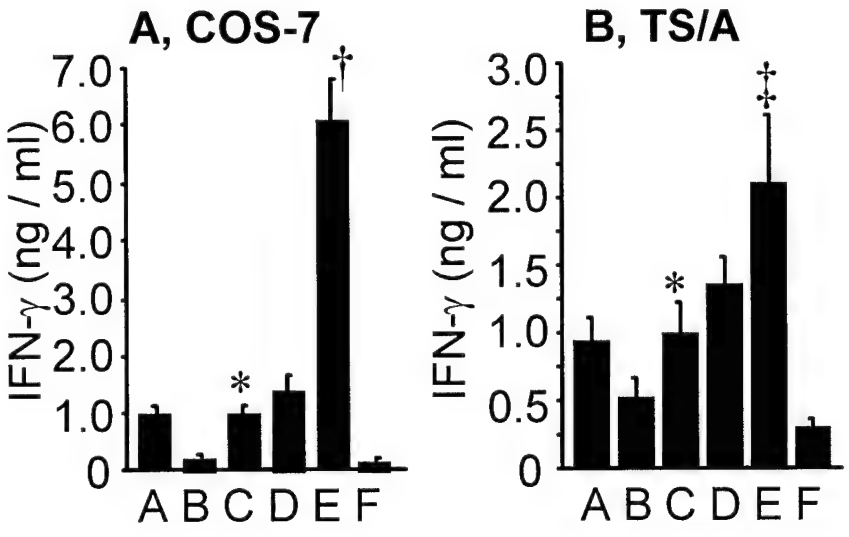


Figure 7. IFN-γ secretion in splenocytes in vitro induced by cytokine proteins secreted from transfected COS-7 (A) and TS/A (B) cells. IL-18 bioassay (stimulation of IFN-γ release) was carried out as described in Materials and Methods. Means ± S.D. were calculated from triplicates. *, P < 0.05 versus pro-IL-18 alone in both cells; †, P < 0.0001 versus the other groups in COS-7 cells; ‡, P < 0.005 versus the other groups in TS/A cells. A, IL-12 c DNA; B, pro-IL-18 cDNA; C, pro-IL-18 / ICE cDNA;D, IL-12 / pro-IL-18 cDNA; E, IL-12 / pro-IL-18 / ICE cDNA; F, control plasmid (pNGVL-3).

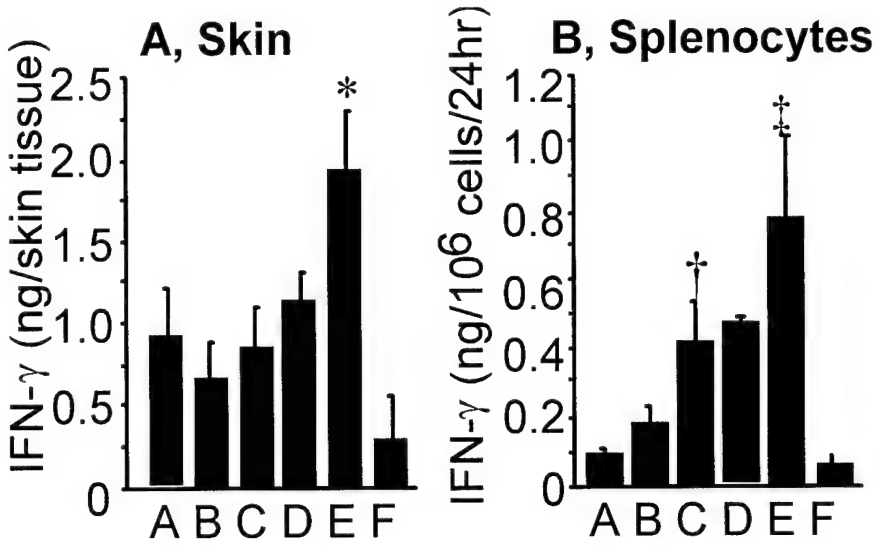


Figure 8. IFN-γ production in the treated skin tissue (A) and from splenocytes (B) after PMGT. A) Skin overlying TS/A tumor was excised after the second gene treatment and IFN-γ in the skin tissue lysate was measured by ELISA. *, $P < 0.05$ versus all other treatment groups. Mean ± S.D. are shown for 4 mice per group. B) Splenocytes (2×10^6) were isolated after the second gene treatment and cultured in 2 ml of RPMI 1640 for 24 hours, †, $P < 0.05$ versus pro-IL-18 alone; ‡, $P < 0.05$ versus all other groups. Mean ± S.D. are shown for 4 mice per group. A, IL-12 cDNA; B, pro-IL-18 cDNA; C, pro-IL-18 / ICE cDNA; D, IL-12 / pro-IL-18 cDNA; E, IL-12 / pro-IL-18 / ICE cDNA; F, control plasmid (pNGVL-3).

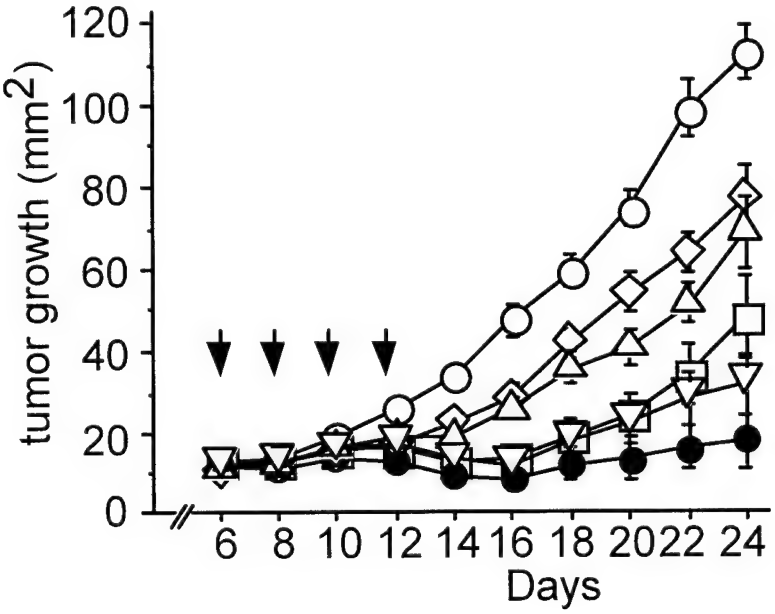


Figure 9. TS/A growth following *in vivo* PMGT. The protocol of PMGT was described in Materials and Methods. Arrows indicate the days when gene treatments were performed. Mean tumor size ± SEM are shown for 15 mice per each group. A statistically significant difference in suppression of the tumor growth was observed in the group treated with IL-12 / pro-IL-18 / ICE, compared with that from pro-IL-18 alone ($p < 0.05$ on day 12, and $p < 0.0001$ on day 14 to 24), from pro-IL-18 / ICE ($p < 0.001$ on day 14 and $p < 0.0001$ on day 16 to 24), from IL-12 alone ($p < 0.05$ on day 22 and $p < 0.01$ on day 24), and from the control plasmid ($p < 0.0001$, day 12 to 24). □, IL-12 cDNA; , pro-IL-18 cDNA; Δ, pro-IL-18 / ICE cDNA; ∇, IL-12 / pro-IL-18 cDNA; ●, IL-12 / pro-IL-18 / ICE cDNA; ○, control plasmid (pNGVL-3).

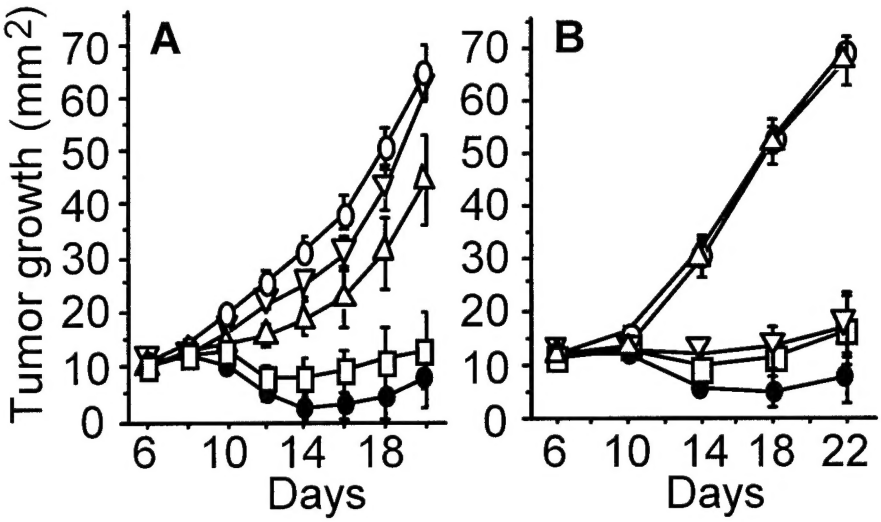


Figure 10. Effects of *in vivo* depletion of $CD4^+$ and $CD8^+$ subsets of T cells and neutralization of IFN-γ on the antitumor response induced by combinatorial gene therapy with IL-12, pro-IL-18 and ICE. A) Mixture of anti-CD4 and anti-CD8 mAb, or anti-IFN-γ mAb were injected intraperitoneally. Mean tumor size ± SEM are shown for 5 mice per group. ∇, anti-CD4 and anti-CD8 mAb; Δ, anti-IFN-γ mAb; ●, no mAb; ; □, rat IgG. All above groups (∇, Δ, ● and □) received IL-12 / pro-IL-18 / ICE cDNA treatment. ○, control plasmid (pNGVL-3) and no mAb. B) Anti-CD4 and anti-CD8 mAb were administered separately. Mean tumor size ± SEM are shown for 6 mice per group. ∇, anti-CD4 mAb; Δ, anti-CD8 mAb; ●, no mAb; □, rat IgG. All above groups (∇, Δ, ● and □) received IL-12 / pro-IL-18 / ICE cDNA treatment. ○, control plasmid (pNGVL-3) and no mAb.

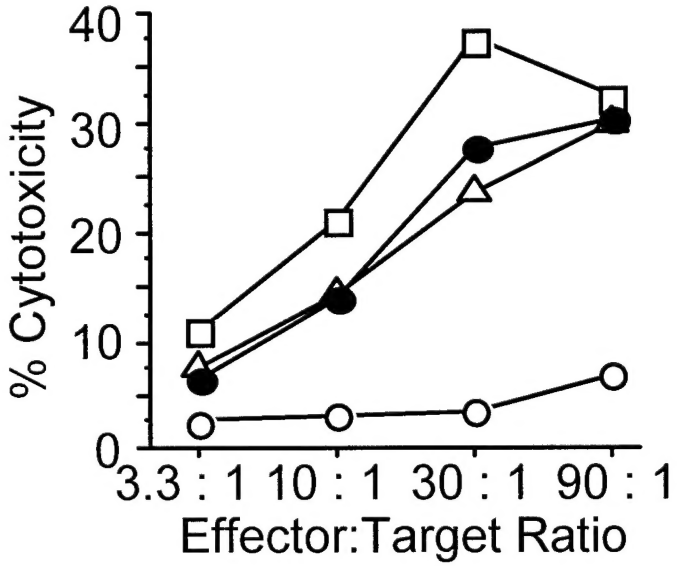


Figure 11. Induction of CTL activities in mice with complete regression was observed following gene therapy with IL-12, IL-12 / pro-IL-18, or IL-12 / pro-IL-18 / ICE. The representative data of two independent experiments are shown. □, IL-12 cDNA; Δ, IL-12 / pro-IL-18 cDNA; ●, IL-12 / pro-IL-18 / ICE cDNA; ○, control plasmid (pNGVL-3).

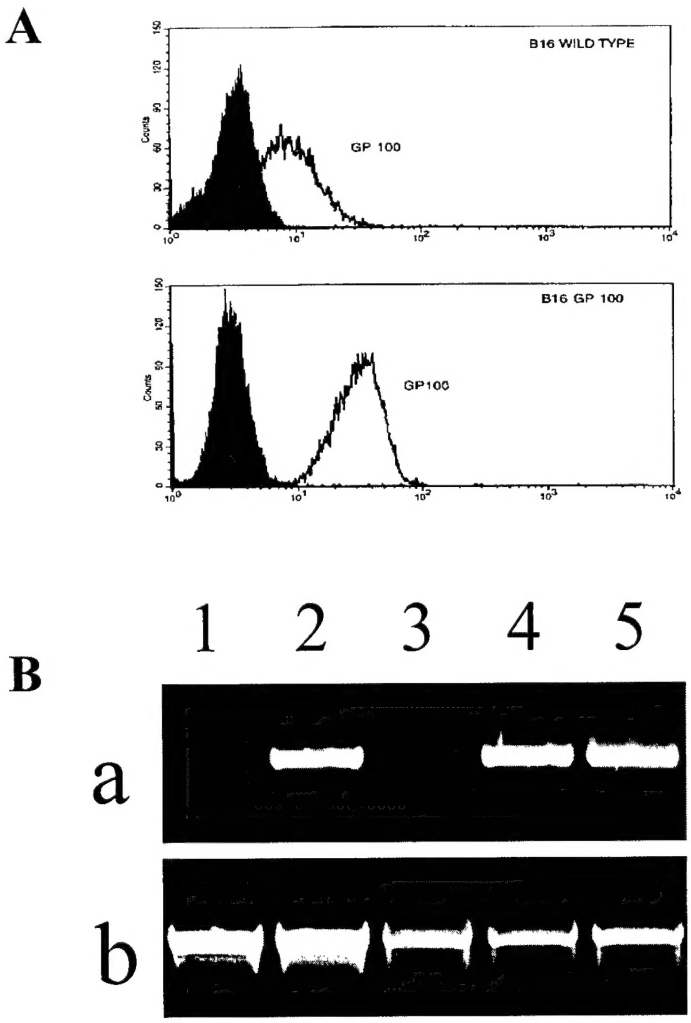


Figure 12. Expression of hugp100 in B16-gp100 melanoma cells and in the skin following vaccination with hugp100 plasmid cDNA. *A.* Mouse B16 melanoma cells (B16-wild), or the cell clone derived from B16 cells following particle-mediated transfection with human gp100 cDNA and selection in G418-containing media (B16-gp100), were used for flow cytometry. The cells were first permeabilized, then incubated with HMB-45 mAb reactive with human gp100, and stained with FITC-conjugated goat anti-mouse IgG. *B.* RT-PCR detection of human gp100 transcripts in the skin after PMGT of hugp100 cDNA. The abdominal skin area of C57BL/6 mice was transfected into with 625 ng of hugp100 plasmid DNA. Twenty four hr post transfection, skin samples were removed, RNA extracted and RT-PCR performed using the primers specific for the hugp100 sequence. *a*, Lane 1, hugp100 transcripts in control B16wt cell line, Lane 2, hugp100 transcripts in B16-gp100 stably transfected cell line, Lane 3, hugp100 transcripts in the skin from a control mouse transfected with empty vector, Lane 4, hugp100 transcripts in hugp100 -transfected skin, Lane 5, hugp100 transcripts in hugp100+GM-CSF transfected skin. *b*, internal control amplification using primers for the house keeping gene G3PDH in all the samples (Lane 1-5).

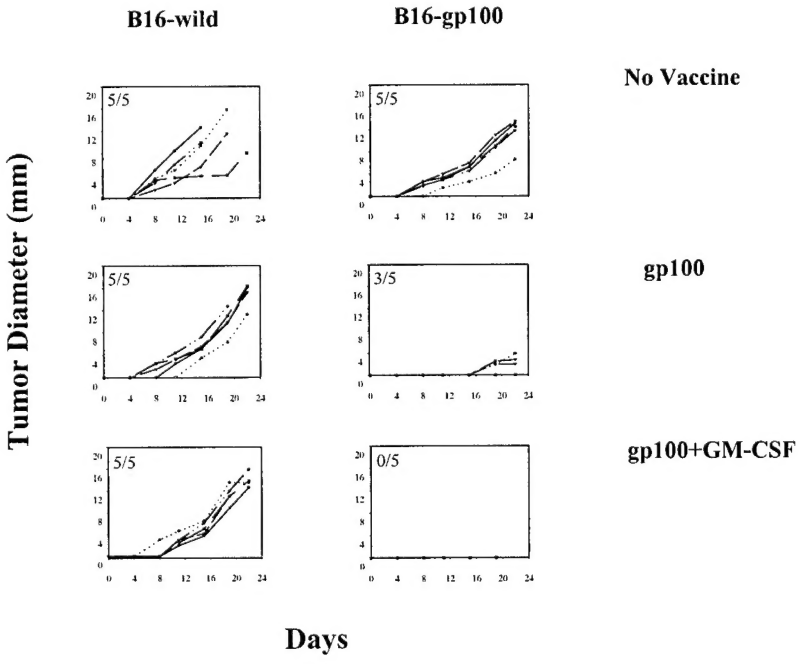


Figure 13. Gp100 gene vaccination: Protection against B16 melanoma. Skin of C57Bl/6 mice was transfected at four sites with hugp100 cDNA (2.5 μg/mouse), or hugp100 cDNA in combination with GM-CSF cDNA (2.5 μg/mouse of each) using PMGT. Seven days later, vaccinated and naive mice were challenged i.d. with $5x10^4$ parental B16 cells (B16-wild), or with $5x10^4$ cells of the B16 cell clone stably expressing hugp100 (B16-gp100). Tumor growth was followed. Data are presented for 5 mice per group. Mice which did not develop tumors remained tumor-free for at least 60 days.

Figure 14. Dose effect of gp100 DNA, alone or in combination with GM-CSF DNA, on tumor protection. Skin of C57Bl/6 mice was transfected using PMGT with hugp100 cDNA (625, 62.5, or 6.25 ng/transfection/mouse) either alone or in combination with GM-CSF cDNA (625 ng). Control mice were transfected with the DNA encoding the empty vector, alone or in combination with GM-CSF DNA. Seven days later, vaccinated and naive mice were challenged intradermally with $5x10^4$ cells of the B16 cell clone stably expressing hugp100 (B16-gp100). Tumor growth was followed. Data are presented for 5 mice per group, where each line represents one animal.

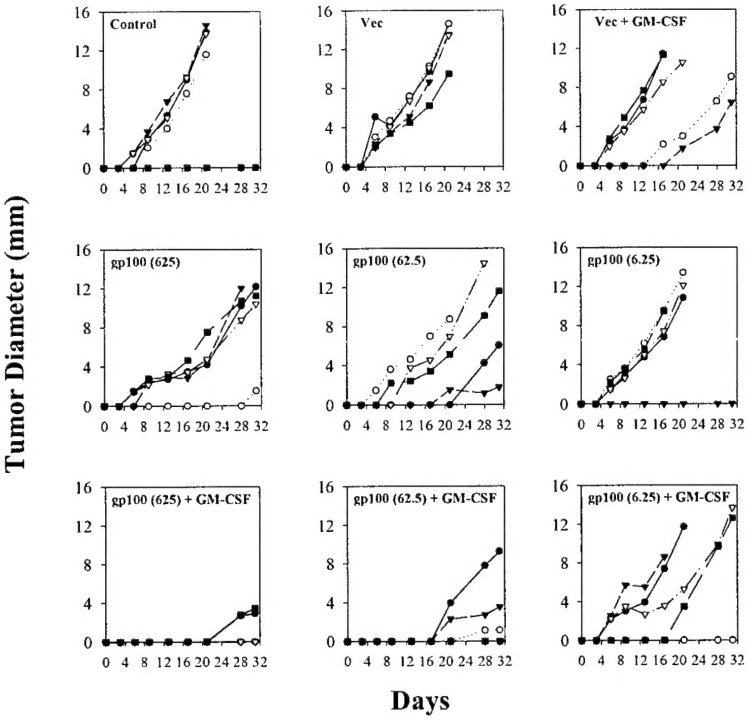
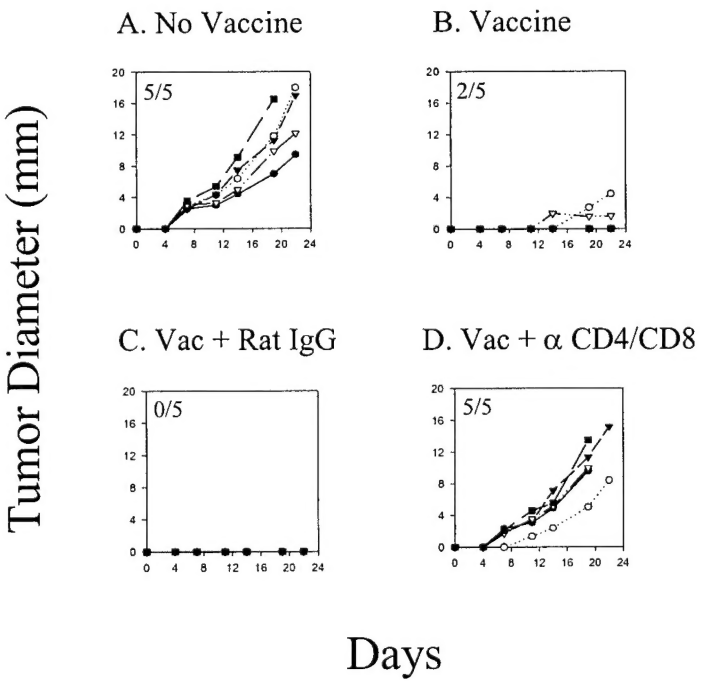


Figure 15. The role of T cells in tumor protection following gp100 + GM-CSF DNA vaccination.
Skin of C57Bl/6 mice was transfected with hugp100 cDNA in combination with GM-CSF cDNA, 0.6 μg/mouse of each (vaccine). A mixture containing 300 μg of each anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) was administered intraperitoneally on days 6 and 11after vaccination. Control groups received rat IgG (Sigma) at the dose 600 μg/mouse per day. On day 7 post vaccination, vaccinated and naive mice were challenged i.d. with $5x10^4$ cells of the B16 cell clone stably expressing hugp100 (B16-gp100). Tumor growth was followed. Data are presented for 5 mice per group.



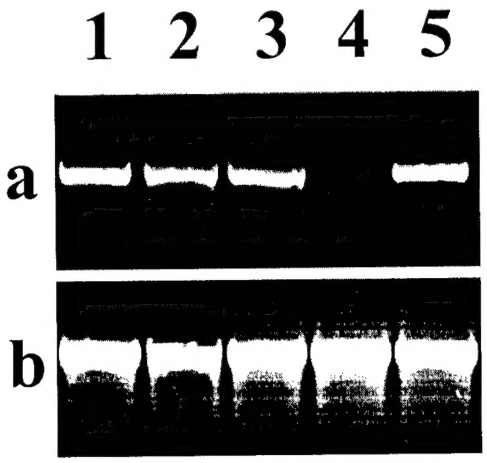


Figure 16. Detection of hugp100 transcripts in recurring tumors after vaccination with gp100 cDNA. Mice were vaccinated in the skin using PMGT with hugp100 cDNA alone or in combination with GM-CSF cDNA. Seven days later, the mice were challenged i.d. with $5x10^4$ B16-gp100 cells. In one experiment, the vaccinated mice that remained tumor-free for 35 days post tumor challenge, were challenged a second time with the same tumor. The intradermal tumors which developed in some vaccinated mice were later removed and analyzed by RT-PCR for expression of hugp100 RNA using hugp100-specific primers. *a*, Lane 1, hugp100 transcripts in the tumor which developed on day 8 after the first tumor challenge and was extracted on day 37. Lane 2, hugp100 transcripts in the tumor which developed on day 26 after first tumor challenge and was extracted on day 37. Lane 3, hugp100 transcripts in the tumor which developed on day 36 after secondary tumor challenge and was removed on day 43. Lane 4, hugp100 transcripts in B16-wild cells, Lane 5, positive control, hugp100 transcripts in B16-gp100 cell line. *b*, internal control amplification of house keeping gene G3PDH in all the samples (Lane 1-5).

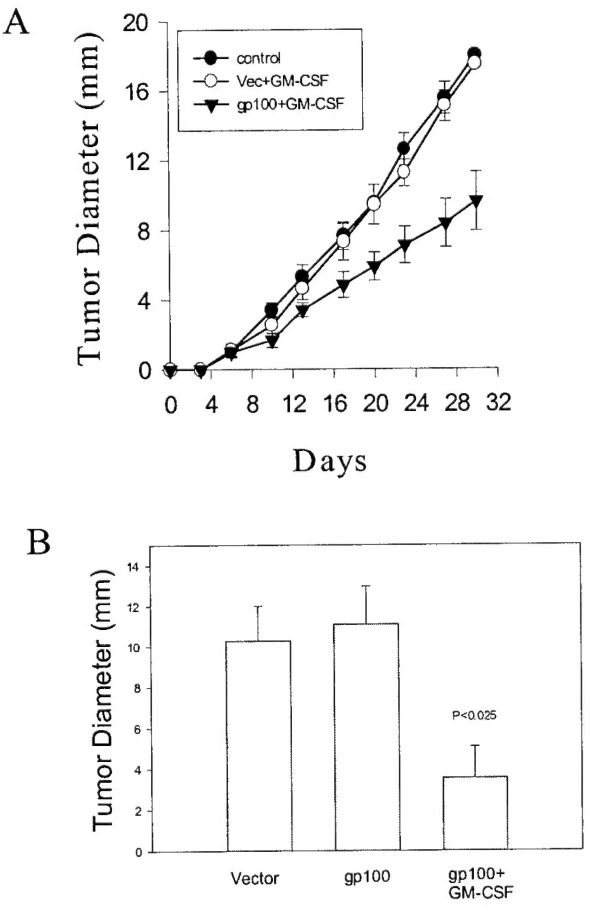


Figure 17. Suppression of the growth of established B16-gp100 tumors, and protection against a wild type B16 tumor, by the gp100+GM-CSF DNA vaccine. A. C57BL/6 mice were injected i.d. with $5x10^4$ of B16-gp100 cells. Seven days later, the skin of tumor-bearing mice was transfected at two sites of the abdominal area using PMGT with hugp100 DNA in combination with mGM-CSF DNA, 625 ng of each plasmid per transfection site. Control mice included untreated mice and mice that received empty vector DNA + GM-CSF DNA. Treatments were continued on day 10,13 and 17. Kinetics of tumor growth was assessed by measuring the tumor diameter. The mean $\pm$ SEM of tumor diameter for all surviving mice at each time point is shown. Each treatment group consisted of 8 mice, whereas the nontreated group consisted of 9 mice. B. Skin of C57BL/6 mice was transfected at four sites with vector or hugp100 DNA (2.5 μg/mouse) using PMGT on days –42, -21 and -7. One group mice was transfected with hugp100 and mGM-CSF DNA (2.5 g/mouse of each) on days –45, -24 and –10. Seven days later (day 0), vaccinated and naïve mice were challenged i.d. with $5x10^4$ wild type B16 cells. Tumor diameters (Mean + SEM) are presented for 5 mice per group on day 18 post tumor challenge.